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LIFT-bioprinting, is it worth it?

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ABSTRACT

To date, laser-induced forward transfer (LIFT) is one of the most developing areas in bioprinting. It is based on a precise nozzle-free laser-assisted hydrogel microdroplet transfer. Although this technique was first mentioned in the 1980s, it started to gain popularity in biomedicine only a decade ago. While the interest in LIFT bioprinting is constantly growing, it is essential to provide a framework of its possibilities and limitations. This review aims to facilitate the search for a common language between physicists and biologists and thus become a short guide to using LIFT technology for biomedicine. Here, we compared various points such as lasers, bioinks components, collector substrate, post-treatment, and printing processes that are crucial for LIFT bioprinting and applied in published studies on it. The core of this review is the discussion of biological and physical aspects to fabricate tissues and organs and the not-known difficulties that can be encountered during the laser printing process and were not given sufficient attention earlier.

1. Introduction

Regenerative medicine has two main options for tissue and organ regeneration: stem cell therapy and tissue-engineered construct (TEC) implantation. The past decade has seen a growing trend towards applying TECs, since they mimic the natural environment for cell growth and differentiation most accurately [1]. Nowadays, three-dimensional bioprinting is widely used in the field of regenerative medicine [2]. Layer by layer, it allows either for the creation of a 3D matrix from biocompatible and bioresorbable materials or for the selective deposition of biological objects (e.g., cells [3–6], drugs [7], growth factors [8], and nucleic acids [9]). Current methods of bioprinting include producing scaffold-based [10] and scaffold-free TECs [11], which have already been used for tissue substitution [12] and for modeling of organs-on-chips [13].

There are three broad methods for TEC bioprinting: inkjet- [14], extrusion- [15] and laser-based [16,17] technologies. The former was the first bioprinting technology [18] and is now the cheapest approach to producing tissue-engineered structures. However, compared to others, this technology has many limitations due to its low resolution and the specific requirements for bioink viscosities [17]. Extrusion-based bioprinters are affordable, easily constructed, and have a wide bioink viscosity range, but they are also seen as having problems with cell viability and printing speed due to using a nozzle [16]. In recent years, there has been an increasing interest in the laser-induced forward transfer (LIFT) technique (Fig. 1), a new and actively developed area of laser bioprinting [19]. The method is based on a nozzle-free laser-assisted hydrogel microdroplet transfer, and therefore it avoids all nozzle-associated negative effects.

Generally, LIFT setups have the same design: the donor ribbon and the collector substrate are mounted onto parallel holders with motorized, computer-controlled translation, which provide their required positioning relative to the laser beam. More advanced laser setups also include galvanometer-based optical scanners (for high-speed laser beam positioning), microscopes (for laser spot visualization), and automatic control systems (for controlling the laser spot size and the energy of each laser pulse). However, to date, there is no commercial alternative to the laser bioprinter NGB-R sold by the French company Poietis; one can purchase it or assemble an experimental laser setup from affordable standard components [20].

Most laser bioprinting techniques provide the necessary resolution

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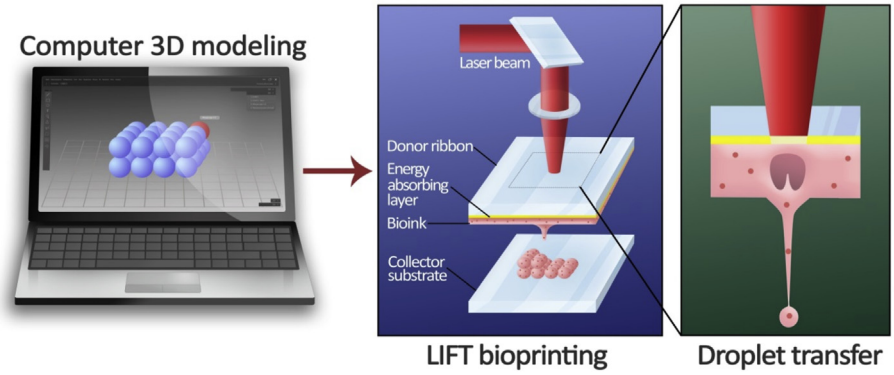

Fig. 1. Schematic of the LIFT bioprinting technique. Applying a software, a computer-aided design (CAD) of the printed structure is converted to the control code, which then initiates the printing process and synchronizes the work of all laser setup elements. The LIFT setup consists of a laser source, a focusing system, a donor ribbon (a transparent glass slide coated with an energy absorbing layer and a bioink), and a collector substrate (a glass slide coated with the hydrogel or any other biocompatible material). The laser beam evaporates the focal area of the absorbing layer generating a vapor bubble that pushes the bioink forward, and the resulting jet or droplet lands on the collector substrate.

and the acceptable print speed [21], however, LIFT is also distinguished by its high adaptive potential, throughput, and cell survival [20]. Moreover, besides cells, this technique has already been used to transfer growth factors [8], nucleic acids [22], and soil microparticles [23,24].

Modern laser sources have regimes for printing a wide range of bioinks. Consequently, the greatest limitation of mastering the LIFT technique is that each hydrogel-cells combination requires a specific selection of appropriate printing parameters. Moreover, the complexity of LIFT technology implementation conditions the importance of having a multidisciplinary team with a clear understanding among research group members. We see great potential in LIFT technology and believe that it should be actively explored by all researchers involved in bioprinting. Therefore, this review aims to describe the main physical and biological issues common for LIFT bioprinting and provide the framework of its possibilities and limitations that will be easy to understand by both biologists and physicists.

2. Methods

A literature search and further selection of studies were performed according to the PRISMA statement [25].

The search was conducted via PubMed and Scopus databases. Subsequently, all relevant papers published on PubMed and included in Scopus until November 1, 2018, were retrieved.

2.1. Search strategy

2.1.1. Pubmed

((((((“Bioprinting"[Mesh] OR biofabrication*) AND (“lift” OR “Laser-Induced Forward Transfer " OR “Laser Induced Forward Transfer” OR “laser assisted” OR “laser printing” OR “laser bioprinting")))) NOT ((((“Bioprinting"[Mesh] OR biofabrication*) AND (“lift” OR “Laser-Induced Forward Transfer " OR “Laser Induced Forward Transfer” OR “laser assisted” OR “laser printing” OR “laser bioprinting"))) AND ((pubmed books[filter] OR Editorial[ptyp] OR Letter[ptyp] OR Meta-Analysis[ptyp] OR Review[ptyp] OR Scientific Integrity Review[ptyp] OR systematic[sb]))))) AND (“0001/01/01"[PDat]: “2018/11/01"[PDat]) AND English[lang])

2.1.2. Scopus

TITLE-ABS-KEY ((bioprinting OR biofarbication) AND (lift OR “Laser-Induced Forward Transfer " OR “laser assisted” OR “laser printing” OR “laser bioprinting”)) AND (LIMIT-TO (DOCTYPE, “ar”) OR LIMIT-TO (DOCTYPE, “cp”)) AND (LIMIT-TO (LANGUAGE, “English”))

2.2. Study selection

Two reviewers (AA & SA) independently analyzed the abstracts for all retrieved studies. In the case of disagreement between the two reviewers, a third reviewer (TP) was consulted. The inclusion criteria were as follows: (1) LIFT technology application; (2) relation to biofabrication; (3) English language. The exclusion criteria were as follows: (1) reviews, editorials, letters, books, and abstracts; (2) duplicates; (3) no cell printing experiments; (4) insufficient data. Based on the inclusion and exclusion criteria, a final list of studies that underwent full-text assessment was made. Subsequently, the selected studies were analyzed in a qualitative manner.

3. Results

Fig. 2 demonstrates the searching and screening processes. The initial search found 105 articles. After removing duplicates from two databases, titles and abstracts were screened to exclude papers that did not meet the inclusion and exclusion criteria. Further process of exclusion required full-text assessment because not all the studies clearly described their methods in abstracts. Finally, 33 studies were selected for the review.

The included studies were systematically analyzed for the types of laser sources, energy absorbing layers, the donor ribbons, and collector substrates. Also, the aims and outcomes of the studies were determined. The collected information is presented in Table 1.

3.1. Lasers

In most studies, two main laser wavelengths were applied: 193 nm [39,40,42,43,46,49,51–53] and 1064 nm [20,27–35,37,38,41,44,45,50,54–57]. Other wavelengths were also tested: 248 nm [26,36], 266 nm [26], 355 nm [48,54], 532 nm [54], 1030 [47], and 2940 nm [56]. Although nanosecond lasers are currently assumed to be the most optimal choice for LIFT bioprinting, microsecond [56], picosecond [54] and femtosecond [47] models were successfully investigated for cell printing as well.

Interestingly, an energy absorbing layer (EAL) (gold [20,27,29–33,35,37,38,41,44,45,47,50,54–57], titanium [28,34,48], gelatin [39,40,46,49,53], or Matrigel® [56]) was not used for cell printing in five studies [42,43,49,51,52]. Xiong et al. [49] demonstrated that a lack of absorbing layer during UV laser printing slightly increased DNA double-strand breaks, but this cell damage was considered negligible.

In their parametric study, Koch et al. [54] investigated the influence of various laser parameters (wavelength, pulse duration, pulse energies) on printing results. It was shown that a wide variety of lasers can be adjusted for LIFT with metal EAL, and the same printing quality will be obtained. However, in the case of printing 3D cornea-mimicking structures consisting of two and more cell types, Sorkio et al. [56] reported that the best decision would be to use laser system with 2940 nm wavelength and hydrogel EAL consisting of Matrigel® and glycerol.

3.2. Bioink components

3.2.1. Hydrogels

Fig. 3 shows the relationships among the hydrogels applied for coating donor ribbons in 33 analyzed studies.

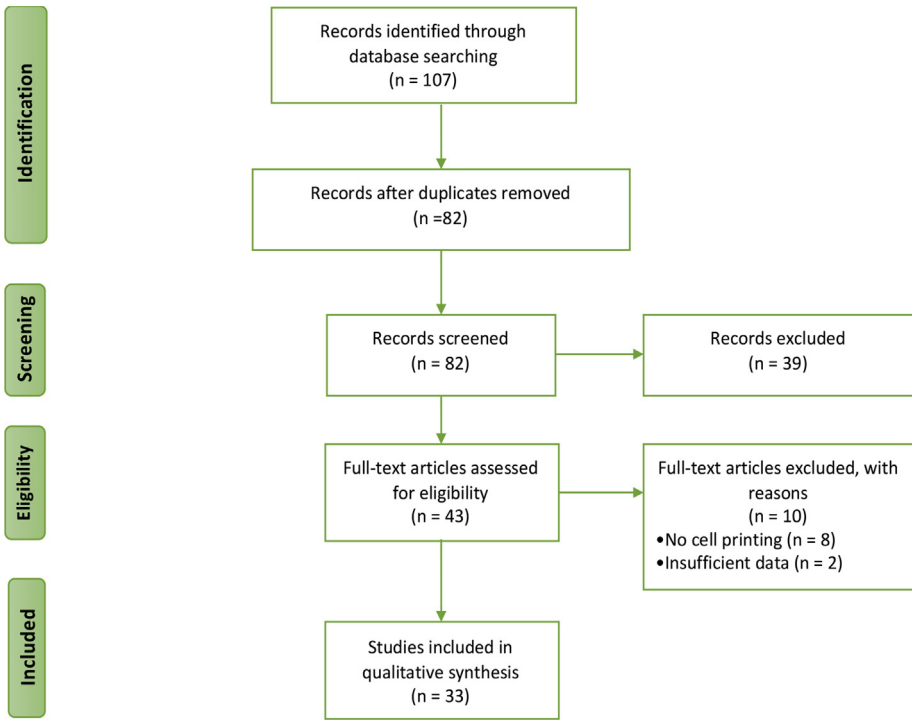

Fig. 2. Flow diagram of included studies.

Generally, bioinks consist of, besides cells, many components, which provide the required physical and biological properties for printing. For example, cell-laden hydrogels demonstrated in Fig. 3 were supplied with glycerol [27,28] and methylcellulose [36] to prevent the bioink drying or with blood plasma [20,29,30,32,37,54,56,57] for supporting cell growth. During the printing process, cell viability on the donor ribbon was maintained by the cell medium which was an obligate component of bioinks in most studies. Of note, the cell medium in Fig. 3 was represented as a separate type of bioinks when it was the only hydrogel component [36,39–41,44–47,50,53,55,57]. Fibrinogen, another bioink component, was always used in combination with hyaluronic acid (HA) [33,57]. However, in two studies, HA was also mixed with laminin [56] or cell medium [57], since it is known to improve bioink viscosity [57]. The group "other" in Fig. 3 includes: thrombin [28], gelatin [48], laminin [56], and Matrigel® [28]. For the latter one, no data about printing quality were presented. Collagen, a natural extracellular matrix (ECM), seems to be one of the best candidates for creating bioinks. In three studies, rat tail collagen (type I) [37,38,57] was used, while in one study [56] human recombinant collagen (type I) was investigated for printing as well.

3.2.2. Cells

Cells are a crucial part of all bioinks, and most of the analyzed studies applied NIH 3T3 mouse fibroblasts [20,37,38,42,43,49,51,52,54] or mesenchymal stromal cells [20,30,32,33,41,45,50,56]. The choice of a particular cell type depended on the study goals and cells availability. As most studies, where LIFT bioprinting is mentioned, discuss the feasibility of this method and optimization of the cell transfer procedure, so the use of the above-mentioned cells seems reasonable as a model. For example, Xiong et al. [43] demonstrated the possibility of fabricating 3D tubes using fibroblasts, Z stage-based receiving platform and supporting crosslinking solution. They especially underlined the importance of choosing an optimal platform downward movement step size (the distance which Z stage-based receiving platform should move downward after the gelation of each newly printed layer). In their paper, Ali et al. [41] discussed how the laser-induced jet dynamics were associated with vertex angle and influenced the MSC printing.

Nevertheless, there are only a few studies aimed at creating a tissue or its structural elements. Lothar Koch was one of the pioneers who printed a multicellular 3D graft and showed the tissue formation by these cells [20]. He and his team fabricated a skin fragment using fibroblasts and keratinocytes encapsulated within collagen and proved the tissue formation via immunohistochemical analysis of Ki-67, laminin, cadherin, and Cx expression and scrape-loading method. Moreover, we can only find a few works where laser-printed cells are used to study migration or invasion. For instance, Vinson et al. [53] printed breast cancer cell-laden microbeads into hydrogel with adipocytes to reveal the cancer cell invasion.

3.3. Collector substrate

Fig. 4 represents the relationships among the components applied for creating collector substrates in 33 analyzed studies.

When the collector substrate contained alginate [30,32,37,53,54] or $CaCl_2$ [39,42,43,49,52], only alginate-based bioinks were used (except one study [57]). In Fig. 4, fibrinogen- [28] and fibrin-based [33,57] collector substrates were joined into one group since fibrinogen, when interacting with thrombin-containing bioink [28] or sprayed with thrombin after printing [33], converts into fibrin. Although human recombinant collagen has already been applied for LIFT as a bioink [56], in the analyzed studies which used collagen for the preparation of collector substrates, it was still isolated from rat tails [41,44,45,47,50,57].

The next collector substrate type is a scaffold or biopaper. For their preparation, either polymeric materials, such as poly(ethylene glycol) diacrylate [29], polycaprolactone [35], polylactic-co-glycolic acid (PLGA) [36], or cells producing their own native ECM [31] were used. Pirlo et al. [36] additionally loaded the polymeric scaffold with Matrigel® layer of different thicknesses, which allowed for the studying of the behavior of cells on its surface in dependence on resulting mechanical properties. The group "other" in Fig. 4 includes: nano-hydroxyapatite (nHA) [34,50], poly(L-lysine)-laminin [46], polyethylene terephthalate [56], Geltrex® [57], and HA [57]. Cell medium was a component of most collector substrates, so it is not shown in Fig. 4 as a separate type of the collector substrate.

Table 1
General information about analyzed studies.

Reference	Laser source	Energy absorbing layer	Bioink*†		Collector substrate†	Aim	Outcomes
			Cell type	Hydrogel			
Wu et al. [26], 2010	248 nm or 266 nm (N/A)	Metal or metal oxide (N/A)	HUVEC (primary) and HUVSMC	N/A	Hydrogel (N/A)	To fabricate a vascular network	Lumen-like branched structures remained intact for at least 9 days
Guillemot et al. [27], 2010	1064 nm	Gold	HUVEC (EA.hy926)	Alginate	Glass slide	To demonstrate the applicability of high-throughput laser bioprinting for 3D tissue fabrication	The authors achieved well-defined cell patterning and nanoparticle deposition
Guillotin et al. [28], 2010	1064 nm	Titanium	a. RCC (B16) b. HUVEC (EA.hy926) c. HUVEC (EA.hy926)	a. Alginate b. Alginate c. Thrombin	a. Glass slide/ Matrigel® b. Glass slide c. Fibrinogen	To print cells with a microscale resolution	Tissue-like layouts with the required resolution were produced
Ovsianikov et al. [29], 2010	1064 nm	Gold	ovEC, ovSMC	Alginate	PEGda scaffold	To combine 2 PP and LIFT techniques	Cell-loaded 3D scaffolds with a distinct architecture were obtained
Koch et al. [20], 2010	1064 nm	Gold	Mouse fibroblasts (NIH3T3), human keratinocytes (HaCaT), hAD- and hBM-MSC	Alginate	Matrigel®	To investigate LIFT's influence on cell survival, proliferation, and apoptosis; DNA and phenotype stability	LIFT technique proved to be a safe technique for cell positioning
Gruene et al. [30], 2011	1064 nm	Gold	hAD-MSC	Alginate	Alginate	To examine the behavior of MSC after laser printing procedure	Laser printing had no negative influence on cells' behavior
Catros et al. [31], 2011	1064 nm	Gold	HUVEC (EA.hy926)	Alginate	Matrigel®	To investigate the influence of printing conditions on cell viability	Cell viability was strongly influenced by the forces occurring at the air–hydrogel and at the hydrogel–glass interfaces
Gruene et al. [32], 2011	1064 nm	Gold	pBM-MSC	Alginate	Alginate	To explore the negative impact of laser printing procedure on MSC differentiation potential	Printed MSC saved their differentiability
Gruene et al. [33], 2011	1064 nm	Gold	hAD-MSC, hECFC	HA-fibrinogen	Fibrin	To create and investigate multicellular arrays	The interaction between MSC and ECFC resulted in the formation of a vascular-like network
Catros et al. [34], 2011	1064 nm	Titanium	HOP	Alginate	Matrigel®/nHA	To arrange HOP on different substrates	2D and 3D patterns of HOP were printed
Catros et al. [35], 2012	1064 nm	Gold	HOC (MG63)	Alginate	PCL biopaper	To evaluate the effects of different spatial arrangements of cells and biopapers on cell proliferation	Layer-by-layer organization (cells-biopaper-cells) had better results than conventional cell seeding onto the scaffold surface
Pirlo et al. [36], 2012	248 nm	Metal or metal oxide (N/A)	HUVEC (primary)	Medium	Matrigel®-loaded PLGA scaffold	To determine printing conditions which influence the capillary network formation of cell-seeded scaffolds	The formation of capillary networks depended on the scaffold rigidity and cell density
Koch et al. [37], 2012	1064 nm	Gold	a. Human keratinocytes (HaCaT) b. Mouse fibroblasts (NIH3T3)	a. Alginate a,b. Collagen	a. Alginate a,b. Matriderm®	To arrange stem cells in the manner of the native skin	The obtained 3D grafts mimicked the structure of the native skin well
Michael et al. [38], 2013	1064 nm	Gold	Mouse fibroblasts (NIH3T3), human keratinocytes (HaCaT)	Collagen	Matriderm®	To produce the native-like skin grafts and to test them *in vivo*	Printed grafts recapitulated the structure of the natural skin and well-integrated into host tissues *in vivo*
Kingsley et al. [39], 2013	193 nm	a. Gelatin-alginate b. Gelatin	a. hBCC (M231) b. hFF	a. Alginate b. Medium	Gelatin-$CaCl_2$	To apply LIFT technique as a single step both to fabricate and pattern alginate microbeads	Fabricated alginate microbeads were arranged with the micron-level precision, while encapsulated cells maintained high viability
Dias et al. [40], 2014	193 nm	Gelatin	mESC	Medium	Gelatin	To control the sizes of embryoid bodies formed from laser printed mESC	The resultant embryoid bodies diameter only depended on the cell printing density
Ali et al. [41], 2014	1064 nm	Gold	mBM-MSC (D1)	Medium	Collagen	To study slow jetting conditions for MSC laser transfer	Slow jetting conditions reduced shear stress within the jet and forces occurred at droplet landing resulting in a high cell viability and high printing resolution
Gudapati et al. [42], 2014	193 nm	No	Mouse fibroblasts (NIH3T3)	Alginate	$CaCl_2$	To explore the effects of laser exposure and alginate gelation process on the viability of printed cells	Higher laser fluence and alginate concentration as well as longer gelation time led to lower cell viability

(*continued on next page*)

Table 1 (*continued*)

Reference	Laser source	Energy absorbing layer	Bioink*†		Collector substrate†	Aim	Outcomes
			Cell type	Hydrogel			
Xiong et al. [43], 2015	193 nm	No	Mouse fibroblasts (NIH3T3)	Alginate	$CaCl_2$	To apply laser printing for the fabrication of 3D vessel-like tubes	Straight and Y-shaped 3D cellular tubes were successfully produced
Pagès et al. [44], 2015	1064 nm	Gold	mBM-MSC (D1)	Medium	Collagen	To demonstrate the capability of LIFT for creating highly defined 3D MSC patterns	3D cellular constructs were printed with the required high resolution and precision
Bourget et al. [45], 2016	1064 nm	Gold	hBM-MSC, HUVEC (primary)	Medium	Collagen	To evaluate the relationships between co-printed EC and MSC	MSC guided the self-organization of EC
Curley et al. [46], 2016	193 nm	Gelatin	rDRG cells, hBCC (M231)	Medium	PLL-laminin	To arrange mammalian neuronal cells into isolated nodes	Printed DRG cells maintained a high viability (close to BCC) and showed the ability to develop the neuronal network
Desrus et al. [47], 2016	1030 nm	Gold	Human keratinocytes (HaCaT)	Medium	Collagen	To apply a low energy LIFT setup for cell printing	Lower printing energy provide higher cell viability
Riester et al. [48], 2016	355 nm	Titanium	CHO cells	Gelatin/ Medium	Matrigel®	To study the jetting behavior for LIFT with the gelatin layer located between the metal EAL and printed bioink	Changes in the gelatin layer concentration significantly influenced the jetting behavior
Xiong et al [49], 2017	193 nm	Gelatin/No	Mouse fibroblasts (NIH3T3)	Alginate	$CaCl_2$	To investigate the effects of gelatin EAL on the printing process	Gelatin EAL improved printing quality and bioink printability and changed the printing mechanism to preferable
Keriquel et al [50], 2017	1064 nm	Gold	mBM-MSC (D1)	Medium	Collagen/nHA-collagen	To investigate different geometries of cell patterning for *in vivo* bone tissue regeneration	Cells patterned in a disk geometry had better regeneration efficacy than cells patterned in a ring geometry
Zhang et al. [51], 2017	193 nm	No	Mouse fibroblasts (NIH3T3)	Alginate	PLL	To study the printing process for cell-free and cell-laden bioinks under the equal laser fluences	Adding cells to the bioink increased the laser transfer threshold energy but reduced the jet velocity, jet breakup length, and printed droplet size
Zhang et al. [52], 2017	193 nm	No	Mouse fibroblasts (NIH3T3)	Alginate	$CaCl_2$	To investigate the cell injury types induced in the laser printing process	One part of injured cells necrotized immediately after printing, while another part committed suicide later through the apoptotic pathway. Some of early-apoptotic cells spontaneously recovered to the proliferative state
Vinson et al. [53], 2017	193 nm	Gelatin	hBCC (MCF-7, M231) encapsulated in alginate-collagen	Medium	Alginate-gelatin-hAD-MSC	To fabricate cell-laden hydrogel microbeads and transfer them into the adipocyte-containing substrate	Deposition of cell-laden microbeads in different spatial arrays allowed for investigating biomimetic models of breast cancer
Koch et al. [54], 2017	355, 532, and 1064 nm	Gold	Mouse fibroblasts (NIH3T3)	Alginate	Alginate/ Uncoated	To find the best LIFT setup for printing with metal EAL	A wide variety of lasers can be adjusted for LIFT with metal EAL, and no universal solution exists
Kawecki et al. [55], 2018	1064 nm	Gold	HUVEC (primary)	Medium	Osseous cellular biopapers from induced hAD-MSC	To explore the influence of self-assembled osseous sheets on the printed human endothelial cells	Cell-based biopapers promoted tubule-like structure formation of endothelial cells
Sorkio et al. [56], 2018	1.1064 nm 2.2940 nm	1. Gold 2.Matrigel® + glycerol	a.hAD-MSC b. hESC-LESC	a. Collagen b. Laminin-HA	1a. Matrigel®/ Matriderm® 1b. PET 2a, 2b. PET/ Matriderm®	To fabricate corneas by using LIFT	Native-like corneas from two cell types were successfully fabricated
Koch et al. [57], 2018	1064 nm	Gold	hiPSC (hHSC_F1285T_iPS2)	Matrigel®/Geltrex®/alginate/ collagen/HA/fibrinogen (used as bioink)/fibrin (as culture substrate)/medium/their combinations		To test different biomaterials for laser printing and study their effects on hiPSC	The best option for printing hiPSC was to use medium + HA as a bioink and Matrigel® as a collector substrate

Notes: * - bioinks without cells are not shown; † - only main components are represented.

Abbreviations:HUVEC - human umbilical vein endothelial cells; HUVSMC - human umbilical vein smooth muscle cells; RCC – rabbit carcinoma cells; ovEC – ovine endothelial cells; ovSMC – ovine vascular smooth muscle cells; PEGda - poly(ethylene glycol) diacrylate; 2 PP- two-photon polymerization; hAD-MSC – human adipose-derived mesenchymal stromal cells; hBM-MSC – human bone marrow-derived mesenchymal stromal cells; pBM-MSC – porcine bone marrow-derived mesenchymal stromal cells; hECFC – human endothelial colony forming cells; HA – hyaluronic acid; HOP - human osteoprogenitors; nHA - nano-hydroxyapatite; HOC - human osteosarcoma cells; PCL – polycaprolactone; PLGA - poly lactic-co-glycolic acid; hBCC - human breast cancer cells; hFDF - human foreskin fibroblasts; mESC - mouse embryonic stem cells; mBM-MSC – mouse bone marrow-derived mesenchymal stromal cells; rDRG - rat dorsal root ganglion; PLL - poly-L-lysine; CHO - Chinese hamster ovary; EAL – energy absorbing layer; hESC-LESC -human embryonic stem cell-derived limbal epithelial stem cells; PET - polyethylene terephthalate; hiPSCs - human induced pluripotent stem cells.

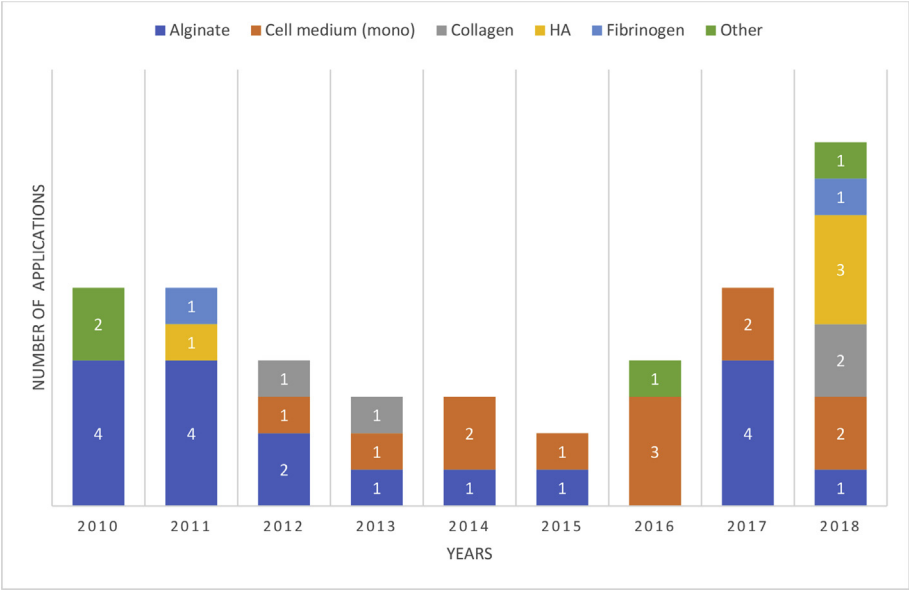

Fig. 3. The number of hydrogel applications for coating donor ribbons by year (2010–2018). Several hydrogel types may be used in one study. HA – hyaluronic acid.

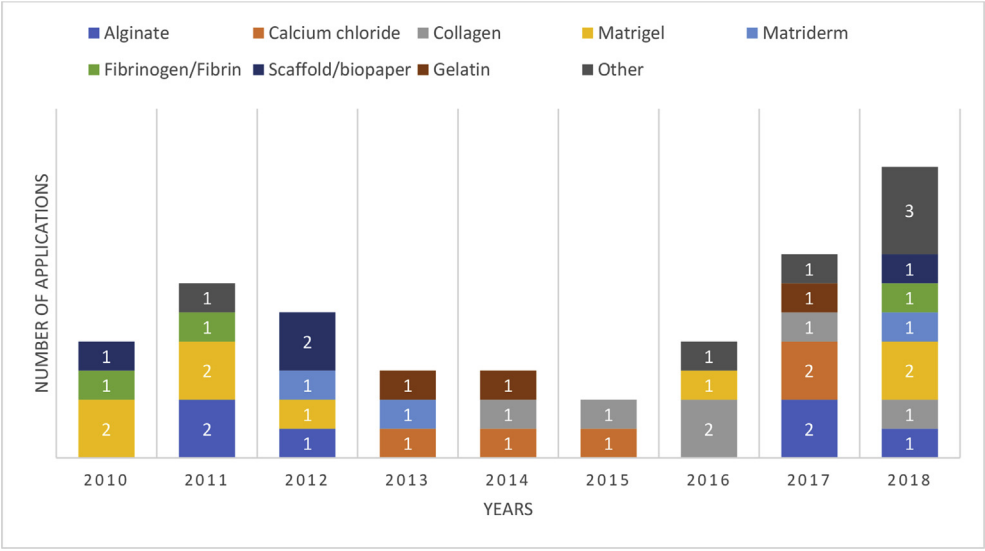

Fig. 4. The number of component applications for creating collector substrates by year (2010–2018). Several components may be used in one study.

3.4. Printing

To distinguish 2D and 3D printing, we consider that a 3D structure consists of two or more bioink layers placed one above the other or of cells printed onto the scaffold/biopaper.

3.4.1. 2D printing

In most studies, the authors printed the cells into 2D patterns as dot matrices or dotted lines. However, cells were also patterned as a number [27], a word [32], or, for example, an Olympic flag [28].

3.4.2. 3D printing

Three-dimensional structures were fabricated in 17 analyzed studies. Wu et al. [26] created a construction from human umbilical vein endothelial cells (HUVECs) and human umbilical vein smooth muscle cells (HUVSMCs) and investigated the relationships between the co-printed cells. The self-assembled HUVEC lumen structure alone degraded in a few days, but when HUVSMCs were deposited onto this structure, they stabilized it for at least nine days. Moreover, HUVSMCs' proximity to HUVECs transformed the former from contractile to proliferative states. The importance of the interactions within the cellular microenvironment was also emphasized by Gruene et al. [30,32,33]. Changing the spatial arrangement, the density, and the ratio of different cell types [mesenchymal stromal cells (MSCs), human endothelial colony forming cells] by LIFT technique, the authors created *in vitro* models of stem cell niches, which are known to play a key role in determining the cell fate by regulating its proliferative potential and differentiation.

It is essential to preserve the projected cellular architectonics when producing complex tissue-engineered constructs. In their study, Pagès et al. [44] showed that after the fabrication of layer-by-layer sandwich constructs consisting of LIFT-printed MSCs covered by collagen layers, the cells in each layer retained their original location, which proved the applicability of LIFT technique for printing with high definition, either in 2D or in 3D. Of note, the same layer-by-layer sandwich construct was fabricated by Keriquel et al. [50], but this study has three special features. Firstly, collagen was mixed with nHA; secondly, the study included tests *in vivo* and the bioprinting was performed directly into mouse calvarial defect; and, thirdly, two cell printing geometries (ring and disk) were tested. For the identical cell

number and density, it was demonstrated that the disk patterning geometry had better regeneration efficacy than the ring patterning geometry, which was explained by better autocrine and paracrine regulation of MSCs. However, for bone regeneration, not only MSCs may be used. Catros et al. [34], found optimal experimental conditions for fabricating the structures consisting of nHA layers and human osteoprogenitors.

The new and original approach to producing 3D structures with LIFT was suggested by Xiong et al. [43,49] and Zhang et al. [51]. In their series of experiments, the formation of 3D cellular alginate tubes was performed with the application of a special Z stage-based receiving platform placed into a crosslinking solution. This printing method allowed for the obtaining of the tubes up to 9.5 mm in height.

Finally, the nature-like structures were produced in the following studies. Koch et al. [37] printed a viable nature-like skin tissue consisting of 20 layers of fibroblasts and 20 layers of keratinocytes. The same skin tissue construction was printed by Michael et al. [38]. Moreover, they demonstrated that the skin constructs fabricated by a laser bioprinter were viable *in vivo* and formed a tissue similar to the native skin. For fabricating the artificial cornea, Sorkio et al. [56] used two cell types constituting its stromal and epithelial component. The stromal component consisted of 60 alternating layers (cellular and acellular), while the epithelial component consisted of three layers. The printed components were positive for collagen and a corneal progenitor marker (p40), thus mimicking the structure of native corneal tissues.

3.4.2.1. Printing with biopapers and scaffolds. In the study by Ovsianikov et al. [29] two laser printing techniques, two-photon polymerization and laser-induced forward transfer, were combined to produce scaffolds and load them with cells respectively. LIFT technique, in turn, yielded a good control over the introduced cell density and location. However, cells should not only be introduced into the scaffold, but also properly arranged within its volume. This topic was investigated by Catros et al. [35]. The authors compared the efficacy of two different cell-scaffold arrangements: a layer-by-layer sandwich model with alternating cell and biopaper layers and a conventional scaffold seeding model with cell layers placed onto the surface of stacked biopapers. Both *in vitro* and *in vivo*, the sandwich-like assembly group showed better results, since it provided better cell immobilization.

Another crucial issue in tissue engineering is scaffold vascularization. Pirlo et al. [36] printed the cells onto PLGA scaffolds loaded with Matrigel® to determine the factors that play a critical role in capillary network formation. The authors proved that substrate rigidity and cell density were the key factors in this process. Interestingly, Kawecki et al. [55] used self-assembled osseous sheets from induced MSCs as living biopapers to study their influence on the printed HUVECs. Living biopapers were considered more preferable than ordinary hydrogel biopapers to create pre-vascularized constructs since the former consist of cells which create natural ECM and produces pro-angiogenic molecules. Indeed, the cell-based biopapers demonstrated the ability to promote angiogenesis *in vitro,* thus proving that such bioprinting technique could be successfully applied for the formation of pre-vascularized scaffolds.

3.5. Post-treatment

Hydrogel gelling procedures were not considered as post-treatment. There were several ways to improve the mechanical properties of the printed construction: spraying them with $CaCl_2$ [30,32,37] or thrombin [33], while Vinson et al. [53] fixed printed microbeads in gelatin by heating the substrate.

4. Discussion

4.1. Biology

Nowadays, LIFT bioprinting is a popular tool for biomedical research. While the number of its applications is constantly growing, it is essential to provide a framework of its typical use in this field and describe the main trends.

However, the main issue common for all bioprinting techniques is that cell viability, behavior, phenotype, genotype, and differentiation potential should remain the same before and after printing. To evaluate the possible LIFT impact on cells, firstly we should check whether the cells remain viable. The common methods used to assess the post-printing cell viability are trypan blue and Live-Dead staining. These simple methods allow us to mark live and dead cells and quickly evaluate their viability via phase contrast or fluorescence microscopy. Many researchers in their studies using various lasers, absorption layers, and cell cultures, have shown that most cells remained viable after LIFT-based printing. Nevertheless, in general, this is insufficient; and in various papers, authors tried to measure additional parameters.

It is common to assess DNA damage using different assays because mechanical forces accompanying the printing process might cause multiple breaks in DNA strands inducing the delayed post-printing cell death. Among these assays, researchers used single-cell-gel electrophoresis (comet assay method) [20], TUNEL assay [44], and immunostaining [49] and showed no DNA damage caused by the printing process. Apart from mechanical forces, cells might be also harmed by high temperature generated locally by laser. Gruene et al. [32] using immunocytochemical staining, analyzed the expression of heat shock protein by the printed cells and revealed no increase.

Nevertheless, the printing process might initiate apoptosis, and cause delayed cell death. To assess apoptosis in the printed cells, the authors measured the caspase 3/7 activity [20,52] and/or detected the phosphatidylserine translocation [52]. Koch et al. [20] tested HaCaT keratinocytes, NIH 3T3 fibroblasts, and human MSCs and revealed no increase in apoptosis compared with non-printed cells up to 48 h after printing. However, Zhang et al. [52] showed that apoptosis in the printed NIH 3T3 fibroblast cells developed gradually and the late apoptotic stage occurred between 4 h and 5 h. These authors used the same cell line (NIH 3T3 fibroblasts), but their methodology was completely different: in the first case, they measured fluorescence using a plate reader after cell lysis and adding *N*-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; and in the second case, they stained cells with annexin V and 7-AAD Apoptosis Kit and Image-iT LIVE Green Caspases 3 and 7 Detection Kit, which contains FAM-DEVD-FMK and propidium iodide. They, then, studied them using a fluorescence microscope. This could explain the difference in the results and show that further investigation is required and the assessment of apoptosis in the printed cells should be carried out together with the analysis of other parameters, first of all, proliferation rate.

In general, the study of the impact on cell proliferation is essential. And many scientists have evaluated how it changed after printing. In their works, they used various methods (water-soluble tetrazolium salt (WST-1) [20] and resazurin [20,44] assays, counting after trypan blue staining [20, 30], photon-imager analysis after adding Beetle Luciferin [35], analysis of Ki-67 expression [37,38] and showed no significant change in the proliferation rate of cell lines and stem cells in comparison with non-printed cells. When stem cells are printed, it is crucial to reveal any effects on their differentiation potential. Immunophenotyping of human MSCs carried out by Koch et al. [20] via flow cytometry showed no significant difference in the ratio of MSC-marker protein (CD29, CD44, CD90, CD105) expression; so the authors stated that the MSC immunophenotype was not influenced by laser printing. The differentiation potential of the printed MSCs was investigated in several studies where the authors revealed no difference between the printed and non-printed cells. The printed cells were able to differentiate toward different lineages: osteogenic [32,44], adipogenic [30], and chondrogenic [32].

Moreover, the growing interest in induced pluripotent stem cells (iPSCs) inspired the assessment of their features after LIFT bioprinting [57]. It is known that iPSCs are more sensitive to manipulation than other cells, with the influence of biomaterials on them being controversial in the literature. Koch et al. revealed that the printing process does not

affect iPSCs' features, but they depend mostly on the choice of material as a base for a bioink. Therefore, iPSCs can be successfully printed without any effects on their potency when bioink containing hyaluronic acid is used. Thus, as most studies claimed no significant change in cell viability, behavior, phenotype, and genotype, LIFT bioprinting may be considered as an appropriate technique for biological and medical applications.

Using LIFT bioprinting, scientists achieved the fabrication of skin and cornea tissue equivalents. The skin equivalents were fabricated from murine NIH 3T3 fibroblast and HaCaT keratinocyte cell lines [37,38]. In the bi-layer construct, the keratinocytes proliferated and formed adherent intercellular junctions; the fibroblasts stayed partly on the top and synthesized collagen or migrated. When the skin substitute was transplanted into mice, blood vessel ingrowth was revealed.

The corneal equivalents were printed with human embryonic stem cell-derived limbal epithelial stem cells (hESC-LESCs) and adipose-derived stem cells (hASCs) [56]. In general, authors fabricated three types of constructs: the corneal epithelium from hESC- LESCs, the corneal stroma from hASCs and acellular layers, and their combination. After printing, the hESC-LESCs maintained the ability to form the stratified epithelial lining and expressed CK3 apically, and corneal progenitor markers p63α and p40 basally; the hASCs formed horizontal layers as in the native tissue and synthesized collagen type I. When the printed stromal structures were transplanted into porcine corneal organ culture model, it was revealed that hASCs attached and migrated into the host tissue in 7 days. The fabricated corneal equivalent from both cell types had moderate transparency, and its morphology was close to the native cornea. There is no doubt that the described tissue equivalents should be improved, but their main aim was to prove the concept of the tissue and organ fabrication via LIFT bioprinting.

Nevertheless, there are few works where testing on animals is described. One of them is the above-mentioned [38] where the printed skin substitute from keratinocytes and fibroblasts were implanted into the dorsal skinfold chamber in nude mice. The other one is the work by Keriquel et al. [50] that describes in situ printing of mesenchymal stromal cells for bone tissue repair. With the improvement in the equipment, the number of publications where animals are involved will rise significantly in the next few years.

Being relatively "young", LIFT bioprinting is only beginning to "conquer" the world of biomedicine. There is no doubt that it will be further improved and used as a tool for tissue engineering, and might even be adapted to clinical use. However, from our point of view, the greatest progress will be achieved when LIFT bioprinting is combined with other fabrication techniques such as scaffolding [58], microfluidics [59], or spheroid formation [60] (Fig. 5). This will allow scientists not only to fabricate vascularized tissues or organs [61], which can be successfully transplanted into humans, but also to develop novel lab-on-chip systems, which imitate native tissues and organs and even a body and can be applied for drug screening, personalized patients' treatment, and fundamental studies of cell-cell, cell-material, cell-cancer cell, and cell-pathogen interactions.

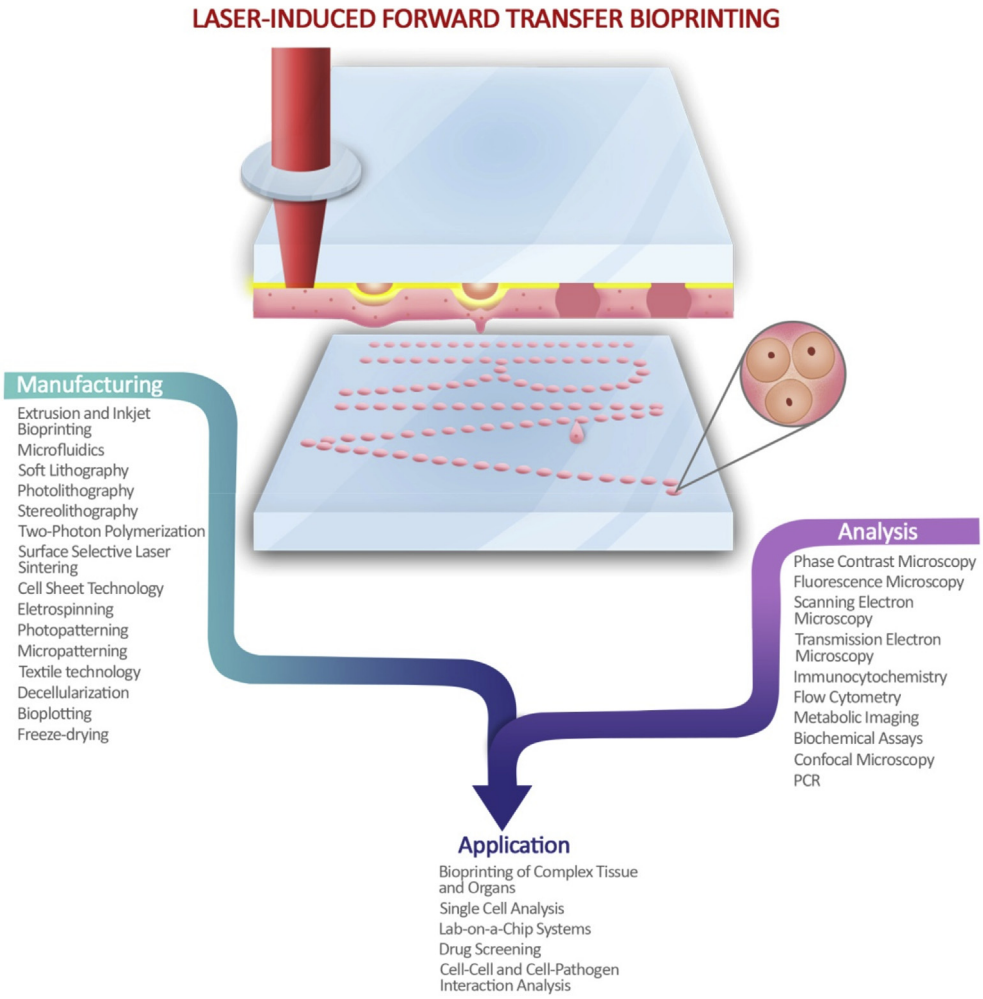

Fig. 5. Prospects of LIFT bioprinting in biomedicine. In the near future, the application of LIFT-bioprinting will totally depend on how it can be combined with analytical methods and other manufacturing techniques. The main area where it can be used focuses on bioprinting of complex tissues and organs, single cell analysis, lab-on-a-chip systems, drug screening, and cell-cell and cell-pathogen interactions.

4.2. Physics

4.2.1. Transfer mechanism

Generally, the LIFT process can be divided into two phases: jet initiation and jetting (Fig. 6). It starts from the focal heating of the energy absorbing layer located between the transparent glass slide and a bioink. For energy absorption, most studies applied metal EAL (usually, gold) with the thickness of 50 nm covered onto a glass slide, but there were also studies using thinner [27] or thicker [57] metal EALs. However, the criteria for choosing gold as a conventional energy absorbing agent were not described in the papers. For example, titanium [28,34,48] has less thermal conductivity than gold [62], and therefore its laser-induced heating could potentially be less harmful to the surrounding bioink. Subsequently, the heated EAL area evaporates generating a vapor bubble, which intensively expands and initiates the jetting process.

There are several typical jetting regimes depending on the laser pulse energy [41]. If the laser energy is insufficient, no material transfer occurs (subthreshold regime). When the required energy threshold is reached, the formed jet has an optimal speed and a single droplet is transferred. A further increase in the laser pulse energy generates high-speed and turbulent jets, which leads to the transfer not only of drops, but also of satellites. Finally, if the optimal laser pulse energy is strongly exceeded, it causes a bioink "explosion" on the donor ribbon surface, and multiple irregular droplets and satellites of different sizes are transferred to the collector substrate. Riester et al. [48] suggested one of the possible ways to control the jet behavior by introducing an additional gelatin layer between the bioink and EAL, which reduced the jet velocity and thereby had a positive effect on the survival of printed cells. Interestingly, after reaching a certain energy threshold, both in the case of printing with gelatin as a bioink and as an intermediate layer, Riester et al. observed the formation of two jets with different velocities from one laser pulse.

It is known that a variety of laser and bioink parameters influence the LIFT process. For bioinks, they are viscosity and thickness [31]. However, most modern bioinks can be printed by LIFT, since this technology has a wide viscosity range [28]. For laser parameters, pulse duration varying from nano-to femtoseconds has the greatest impact on the laser transfer mechanism. For equal pulse energies, shorter pulse duration leads to a smaller volume of the transferred droplet thus increasing the printing resolution. Moreover, in this case, the formed jet is more stable and less sensitive to energy fluctuations, which significantly increases the reproducibility of the results [63].

One other important aspect in LIFT technique is the need for a jet-collector substrate contact. When the jet-collector substrate contact occurs, high jet speed may lead to bioink splashing, while droplet formation before the jet-collector substrate contact, may lead to its excessive spreading after landing [64]. If the printing process is carried out without time-resolved imaging, it will be difficult to determine the true reason for droplet changes, since the laser pulse energy also directly affects the shape and the size of the droplet [51].

To conclude, by selecting a required set of printing parameters, one can provide the conditions for both single and multiple cell transfers, which allows for a variety of printing modes, for example, with high throughput [27,28] or with high precision [48,65].

4.2.2. Laser sources

Nanosecond lasers are more economically accessible and commercially available than others, and, moreover, they have been widely studied by the scientific community. Nevertheless, femtosecond laser sources are more perspective than nanosecond ones. They allow for the minimizing of the effect of bioink local heating [47] and for the converting of the laser pulse energy to the transfer energy more efficiently due to non-linear effects of energy absorption [63]. In a study by Desrus et al. [47], femtosecond lasers at 1030 nm demonstrated the ability to print without EAL, which required increased bioink layer thickness and higher laser pulse energies. It was also shown that the jet form and velocity could be stabilized by changing the laser focusing position and

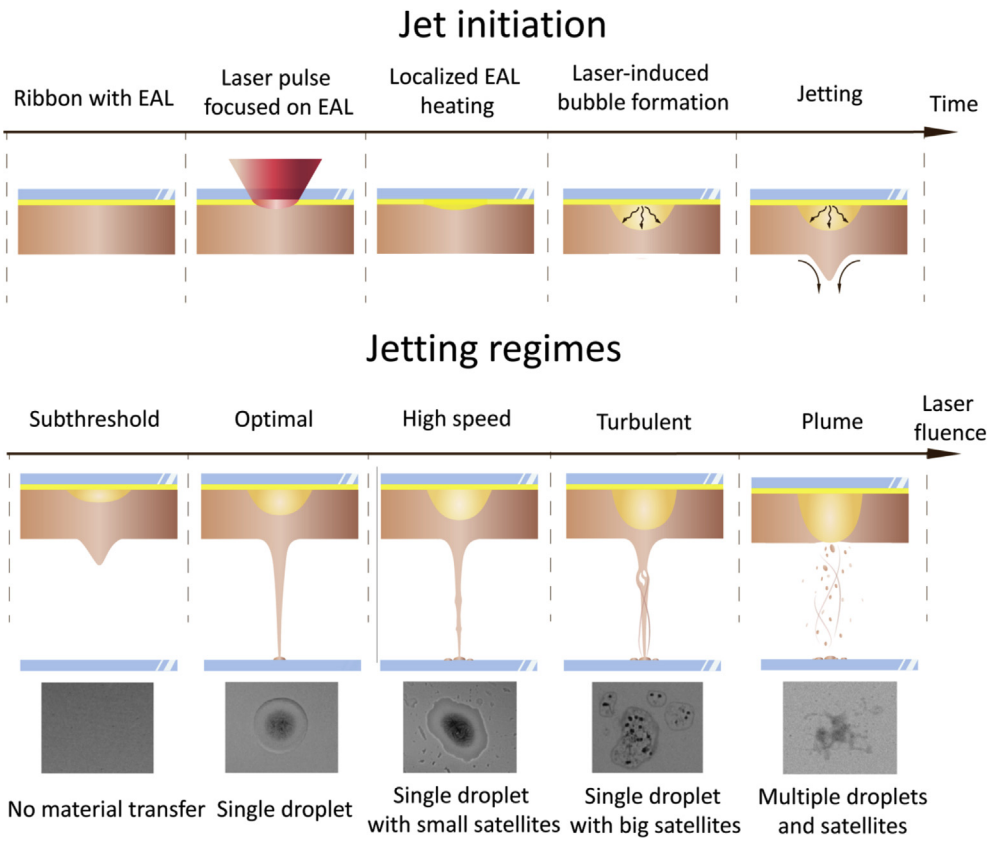

Fig. 6. Schematic of the LIFT process.

numerical aperture. Furthermore, infrared (IR) femtosecond lasers are capable of inverted printing process [66]. In this case, the resulting jet is directed upward perpendicular to the surface of the donor ribbon, which can be advantageous when working with certain types of hydrogels. However, inverted printing is not widely used since its realization is technologically complex.

The radiation of ArF excimer lasers at 193 nm wavelength is efficiently absorbed in micron-thick layers of most hydrogels. This advantage has already been applied in the studies for substituting conventional metal EAL with hydrogel EAL [39,40,46,49,53] or for EAL-free printing [42,43,49,51,52]. The latter bioprinting approach can be potentially detrimental to cells, but we found no evidence of this fact in the analyzed studies.

Nd:YAG infrared nanosecond lasers at a wavelength of 1064 nm can be a simple starting point for investigating LIFT bioprinting. Such lasers are not only economically affordable, but also biologically safe. Infrared lasers are well absorbed by metal EAL (e.g., gold or titanium) about 50 nm thick, while living tissues and, possibly, printed cells are transparent for their radiation [67]. Moreover, it was shown that IR lasers have no effect on the physicochemical properties of bioinks [44].

Of note, another perspective approach to LIFT bioprinting implies using the lasers at a wavelength of 2940 nm. Their energy is almost completely absorbed in the water [68], which allows for printing with hydrogel EALs [56] (for example, Matrigel®) or for avoiding the application of EALs at all. For the latter, the role of EAL is played by a thin layer of the bioink [69] at the glass-bioink interface, which minimizes the chance of cell damage.

Finally, laser wavelengths (248, 266, 355, and 532 nm) have been successfully applied for laser transfer process as well, but they had no advantageous effects compared to commonly used wavelengths [26,36,48,54].

4.2.3. Hot topics

In the analyzed articles, there was no evidence that the researchers had any substantial problems with LIFT bioprinting. In our opinion, there are some hot topics that should be discussed before using this technology.

1. In all analyzed studies applying metal EAL, there were no data on the quality of its deposition (e.g., roughness or stability), which can substantially influence the reproducibility of the laser transfer process. Moreover, the radiation of IR lasers at 1064-nm may penetrate EAL [70] and damage the objects in the coating of the donor ribbon or collector substrate. In addition, laser evaporation of metal EAL leads to the formation of nano- and microparticles. These particles are subsequently transferred onto a collector substrate, where they can be potentially toxic to the living cells [70].

2. As a result of LIFT, cells are transferred onto the collector substrate usually coated with organic materials. Depending on the laser pulse energy, cells penetrate the collector substrate coating on different depths, and therefore it is essential to select the sufficient thickness for the collector substrate coating to prevent cell damage [31,71]. Moreover, even if the collector substrate has sufficient thickness, the depth of cell penetration into the coating still plays a major role in cell viability. Koch et al. [57] showed that when iPSCs were transferred into Geltrex® layer instead of being transferred onto it, their viability decreased [57]. Even though the authors could not explain this, one should consider that transferring cells into the thickness of coatings, which are generally considered to be non-toxic, can still negatively influence cell viability.

3. The jet dynamics directly determines the quality of laser printing, which is especially important when printing complex 2D or 3D structures. Laser pulse energy plays a key role in this process. Zhang et al. [72] distinguish five mechanisms of hydrogel transfer depending on laser pulse energies. When the pulse energy is too low, no jet formation occurs but when the pulse energy is too high, an “explosion” occurs on the bioink surface. There are three mechanisms amongst these regimes that are acceptable for cell transfer: (1) droplet-impingement printing, (2) jet-impingement printing with multiple breakups, and (3) jet-impingement printing with a single breakup. Jet-impingement printing, in turn, includes the formation of slow, high speed, and turbulent jets [41]. Slow jet printing is considered to be the most suitable option since it is more stable and reproducible. High-speed printing and

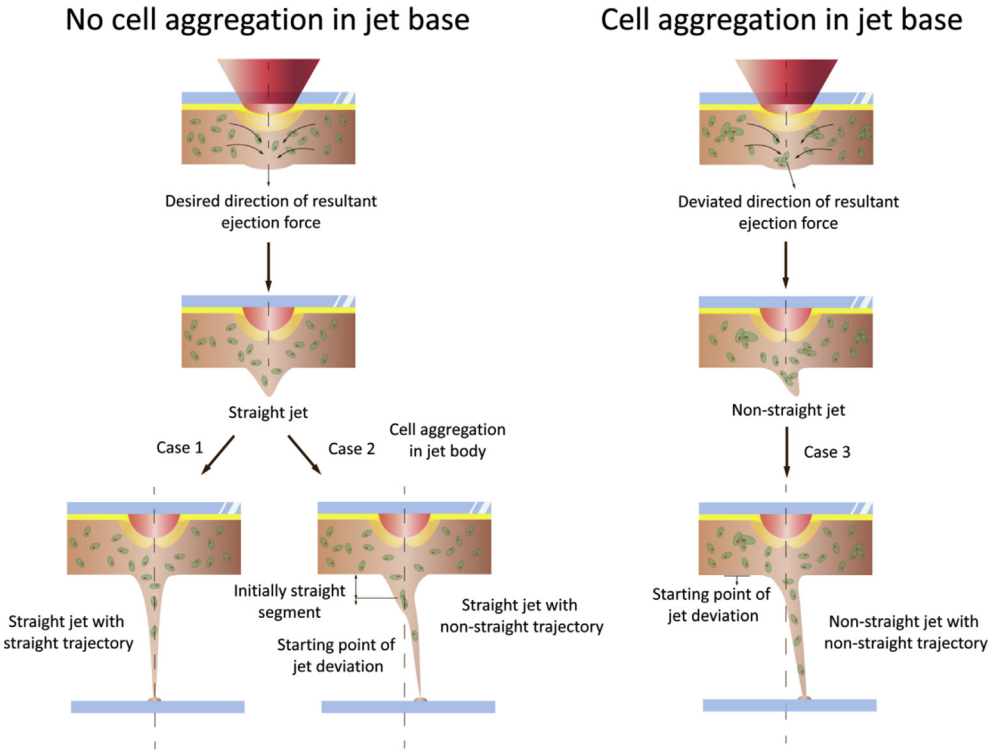

Fig. 7. The effect of cell aggregation on the jet trajectory. Cell agglomeration may occur both during initiating and jetting phases of LIFT process in the jet base and body respectively, which deviates jet from the desired trajectory. Reproduced from Ref. [51], with the permission of AIP Publishing.

turbulent printing are less preferable because jet velocities are higher, and this can lead to droplet splashing [71]. Moreover, the printing of cell-laden bioinks, especially with high cell density, can be complicated by the cell agglomeration [49], which leads to a deviation of the initial jet trajectory (Fig. 7).

4. Obviously, the selection of laser printing parameters, which provide the optimal jet dynamics for each bioink type, is a time-consuming process requiring thorough tuning. Bioink drying is one of the factors that can upset the balance of printing parameters. If the printing is carried out under room conditions, the bioink characteristics tend to change over time (the volume and, as a consequence, the cell density, the thickness, the viscosity), while it significantly affects printing quality, since initially laser parameters were adjusted to different bioink properties. However, to avoid this problem, only two analyzed studies [26,47] controlled the air conditions, while three other analyzed studies [27,28,36] reported adding the supplements which prevented the bioink drying. Preventing the bioink from drying is especially important when printing large 3D structures since the laser printing process can take up to 180 min [49]. Therefore, a high-speed camera with a macro lens will be a good option to control the process of laser transfer: real-time visualization of the jet shape allows for a quick assessment and adjustment of the printing mode.

5. Conclusion

Thus, LIFT bioprinting is a promising approach to the controlled transfer of various biological objects with microscopic precision and resolution. Although this technique was first mentioned in the 1980s, it started to gain popularity in biomedicine only a decade ago. As for any other new technology, at first, it was proven that LIFT was safe for stem cells and it had no effect on their behavior. Subsequently, in recent years, there has been a growing body of works aimed at printing certain living structures (nervous and bone tissues, the vessel, the skin, the cornea) or studying the interaction of different cell types (smooth muscle, endothelial, mesenchymal, and cancer cells) using this technology. However, the number of publications devoted to LIFT bioprinting is still relatively small. This is due to the complexity of LIFT technology implementation coupled with sole commercial laser bioprinter appeared on the market only in 2018. In spite of this, as previously mentioned, one can assemble LIFT setup in the laboratory from the commercially available components.

In this review, we have also shown the not-known difficulties that can be encountered during the laser printing process (ink drying, cell aggregation, potential metal EAL toxicity, etc.) and which, in our opinion, were not given sufficient attention in the analyzed studies. Moreover, there are many fundamental questions about LIFT technology that remain open. Is the jet-collector substrate contact needed? What energy absorbing should be used, and, whether it should be applied at all? What hydrogels and their combinations are best suited for laser printing? Nevertheless, currently, there is already a successful example of applying LIFT technology for the production of commercial products - the company Poietis. In the near future, such companies should inspire the researchers to pay more attention to LIFT bioprinting and create their own startups based on this technology.

Finally, considering current progress in mastering LIFT technique and the results yielded, one can definitely assume that the interest towards this technique will only increase. We strongly believe that LIFT bioprinting is worth it and we hope that this review will become a starting point for researchers who have not yet decided to apply LIFT for their experiments.

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Appendix A. Supplementary data

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