Biological cell printing technologies

Alan Faulkner and Wenmiao Shu*

Institute of Biological Chemistry, Biophysics and Bioengineering, School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, EH14 4AS, UK

In the field of bioengineering, there is a lot of ongoing research with the aim of fabricating cellular constructs with very fine spatial control of cell location. Precise cell placement can enable research thought previously to be impossible, such as cell-by-cell assembly of complex biological structures, including whole organs. Over the years many different techniques have been developed and tested. These techniques employ an extremely varied collection of printing methods including those based on laser pulses, inkjets and other more novel approaches. However, most research has been geared towards developing techniques for accurately positioning viable cells rather than applying the technology to the wide range of possible uses. Here we present a review of the different cell printing technologies that have been developed over the years. The strengths and limitations are evaluated and the techniques are compared and contrasted. A number of applications for cell printing are described.

Keywords: cell printing, micro-patterning

1. Introduction

> *"Every contrivance of man, every tool, every instrument, every utensil, every article designed for use, of each and every kind, evolved from a very simple beginning."*
>
> Robert Collier

Biological cell printing is a relatively new technology in the field of bioengineering. It can be defined as the process of quickly and reliably positioning viable biological cells in predetermined patterns.

Printing technology has come a long way from its origins over 5000 years ago in Mesopotamia as a means of recording information on clay tablets [1]. By the 15th century printing had become quite widespread due to the widespread availability of paper, which spurred advances from simple woodblock printing to the much quicker and more durable moveable type printing press [2]. Up until that point printing technology had been purely

* Corresponding author. E-mail: w.shu@hw.ac.uk

mechanical in nature, but in the 18th century a new process called lithography was invented [3], which uses hydrophobic chemicals to repel the ink solution from the negative areas of the image, thus creating the first method for printing on a smooth surface.

Fast-forward to the late 20th century and the modern, computer-based printing techniques have been developed including the photocopier, laser and inkjet printers. The photocopier was invented by an American office worker named Chester Carlson; it operates on the principle of static electricity, attracting toner to a drum before transferring it to the paper [4]. Laser printers were a direct descendent of the photocopier (xerography) technology but instead of using natural light to determine patterns of toner on a photosensitive drum, a scanning laser beam is used to neutralize the charge on the drum [5]. Inkjet printing technology is divided into continuous inkjet and drop-on-demand, the latter being further divided into thermal and piezoelectric types. The basic premiss of the inkjet techniques is to apply an electrical signal to a heating pad or piezoelectric material triggering the production of a single drop of ink (in the case of drop-on-demand) or a stream of droplets (in continuous inkjet) [6].

It is true that printing technology has come a long way, but it doesn't stop there. Just before the turn of the millennium, an article was published that laid the foundations of a completely new field using the techniques of printing to pattern biological materials [7]. The authors used a modified optical tweezers technique to gently nudge cells in the required direction, but even at that early point it was clear that the authors grasped the power that this new technology could grant: *"the ability to organize cells spatially into well-defined 3D arrays that closely mimic native tissue architecture can potentially help in the fabrication of engineered tissue"* [7, 8].

Only a few years later this exciting new field, now widely referred to as cell printing, had emerged with experiments being conducted worldwide [8, 9]. Until very recently the majority of the research being undertaken was focused on "proof-of-principle" of several different printing techniques including those based on laser pulses, inkjets and other more novel approaches.

Construction of thick tissues such as muscle via cell printing is not currently possible due to the inability to include the intricate vascular system required to ensure that every cell in the tissue is no further than ~1 mm away from a source of nutrients and oxygen within the engineered tissue mass [10, 11]. Therefore, tissues created using cell printing have been limited to thin tissues such as skin, which is exactly what the Wake Forest Institute for regenerative medicine is doing: printing skin over open wounds with simple inkjet valves attached to an *xyz* plotter [12, 13]. So much has already been achieved, but what does the future hold for this new field?

The goal of this article is to present the results of a literature review exploring the concepts of biological cell printing. A number of different approaches for printing viable biological cells into programmed patterns are examined, including traditional and more modern techniques. The methods of each technique are described and their advantages and limitations are listed. The review is concluded by comparing the different techniques.

2. Laser-based direct-write techniques

Laser-based additive writing was originally used to create mesoscopic electronic components, such as conductors, capacitors and resistors with a high spatial accuracy of ~1–3 μm [14]. Thanks to this high accuracy laser-based direct-write techniques became extremely attractive to the fields of biomedicine and bioengineering.

There are several variations on the standard laser-based direct-write technique; the most prolific techniques for cellular applications are *matrix-assisted pulsed laser evaporation direct writing* (MAPLE DW) [15, 16], *biological laser processing* (BioLP) [15, 17, 18], *laser-induced forward transfer* (LIFT) [15, 19–21], *absorbing film-assisted laser-induced forward transfer* (AFA-LIFT) [9, 15], and *laser-guided direct writing* (LG DW) [15].

With the exception of LG DW, these techniques operate in distinctly similar ways [15] (Figure 1). Each of them utilize a laser-transparent ribbon, usually glass or quartz, the underside of which is coated with cells that are uniformly suspended within a thin layer of cell culture medium mixed with glycerol to give a cell concentration of around 1×10^9 cells/mL and a coating thickness of approximately 10–100 μm [16, 17]. A receiving substrate is coated with 50–200 μm of cell culture medium to maintain cellular viability [17, 18], mounted on a computer-controlled motorized stage and positioned beneath the ribbon facing the cell-coated side. In order to transfer the cells from the ribbon to the substrate a pulsed laser beam is fired at the transparent ribbon. The energy of the laser passes through the ribbon and causes the rapid volatilization of the cellular support layer creating the necessary force to allow the cells to drop the small distance (30–1000 μm [20]) between the ribbon and the receiving substrate (Figure 1). The amount of biomaterial, including cells and suspension, that is transferred can be expressed as a function of the focused laser spot size, the thickness of the biomaterial layer on the target, and the laser fluence [16, 20].

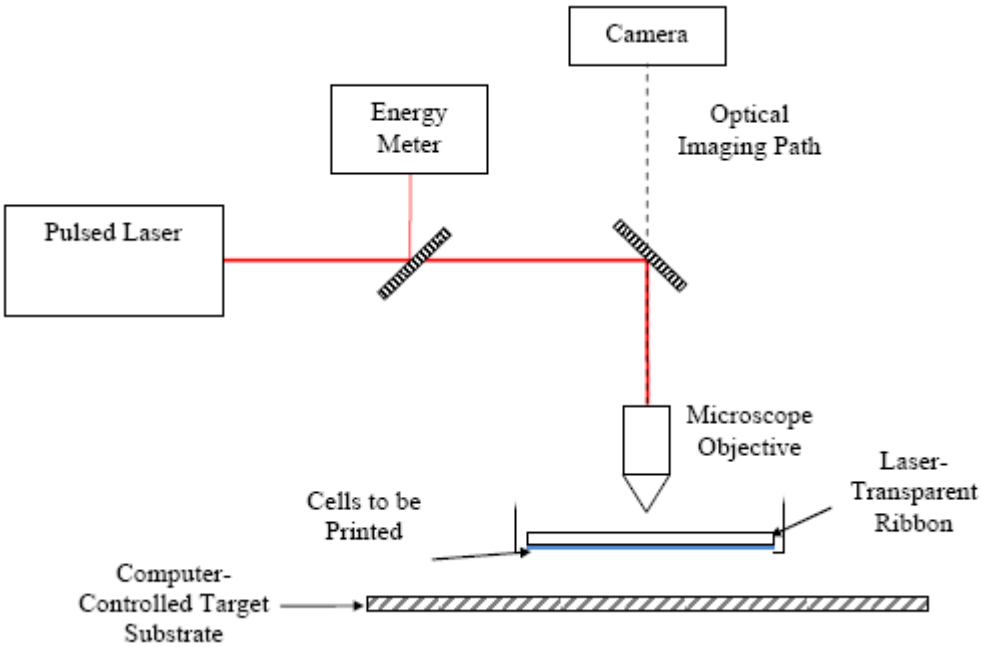

Figure 1. Simplified schematic of a LIFT, AFA-LIFT, MAPLE DW or BioLP system used for cell printing (adapted from [15]).

2.1 LIFT

Laser-induced forward transfer (LIFT) [15, 19, 20, 22] uses a high-powered pulsed laser. Guillotin et al. used a Nd:YAG crystal laser (Navigator 1, Newport Spectra Physics) with wavelength of 1064 nm, 30 ns pulse duration, 5 kHz repetition rate and 7 W mean power [19]. In addition to the higher powered laser, LIFT incorporates a thin (~50–60 nm [19, 20]) coating of a laser-absorbing biocompatible material such as Ti, TiO_2, Au or Ag [15] to the laser-transparent ribbon in order to protect the cells from the laser pulses (Figure 2).

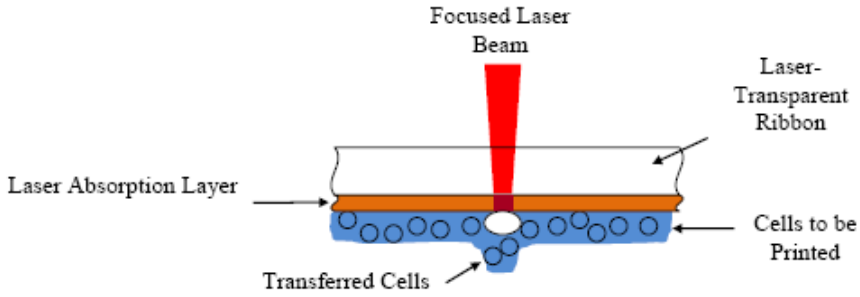

Figure 2. Simplified schematic of a LIFT ribbon arrangement (adapted from [15]).

2.2 AFA-LIFT

Absorbing film assisted laser induced forward transfer (AFA-LIFT) [9, 15] uses the same arrangement as LIFT but instead of a thin layer of laser-absorbing material, a thicker (~100 nm [15]) sacrificial layer of metal is used to interact with the laser. Hopp et al. used a KrF excimer laser with wavelength of 248 nm, 30 ns pulse duration, laser fluence between 35 and 2600 mJ/cm^2 and a 50 nm thick layer of silver [9].

2.3 MAPLE DW

Matrix-assisted pulsed laser evaporation direct writing (MAPLE DW) [15, 16, 18] is a slightly different technique compared to the previous two, the major difference being that MAPLE DW employs a low-powered pulsed laser operating in, or near, the ultraviolet (UV) range of the spectrum. Barron et al. used an ArF excimer laser (Lambda Physik LPX-300i) with 193 nm wavelength, 20 ns pulse duration, laser energies between 15 and 30 μJ/pulse and laser fluences between approximately 157 and 315 μJ/cm^2 [16]. The other difference that sets this method apart is that instead of thin laser absorption or sacrificial layers, the underside of the laser-transparent ribbon is coated with an aqueous biological support layer, typically composed of a laser-absorbing biopolymer and cell attachment layer. The UV light from the laser is absorbed by the water, causing vaporization of some of the liquid at the interface in the biological support layer, which results in ejection of the material below [18].

2.4 BioLP

Biological laser printing (BioLP) [15, 18, 21] is the most recent adaptation of the now classic laser-based techniques. The technique is similar to LIFT and MAPLE DW, but utilizes a laser-

absorbing interlayer rather than the biological matrix support used in MAPLE DW [17, 18]. This absorption layer is typically composed of a titanium or a titanium oxide coating approximately 75–85 nm thick [17]; like the layers used in the other techniques, it prevents the laser from interacting with the biomaterial but also improves the reproducibility of transfer by normalizing the laser interaction [18]. An example of a laser system used for BioLP is the one described by Barron [17, 18]—a quadrupled Nd:YAG (Continuum Mini-Lite) with 266 nm wavelength, 5 ns full width half maximum (FWHM) pulse duration, 1–15 Hz pulse repetition rate and laser fluences approximately 191–382 mJ/cm^2.

2.5 Summary (Table 1) of laser-based techniques

Table 1. A comparison of the different laser-based techniques in this review.

	Laser	Laser fluence/energy	Spot size or resolution (μm)	Cell viability (%)	Advantages/ limitations
LIFT	Nd:YAG λ = 355 nm	65–400 nJ	10	~98	Extremely small droplets possible
AFA-LIFT	KrF Excimer λ = 248 nm	355 mJ/cm^2	~10	75	
MAPLE DW	ArF Excimer λ = 193 nm	157–315 μJ/cm^2	80–100	100	Laser radiation transferred to cells
BioLP	Nd:YAG λ = 266 nm	191–382 mJ/cm^2	30–120	100	<1% laser radiation transferred to cells

2.6 Strengths and weaknesses

Laser-based direct writing (LBDW) techniques have several advantages over others. As the former use an orifice-free transfer process they are unaffected by biomaterial adhesion and therefore easily cope with variations in the viscosity of the biological material [18]. Other advantages include an extremely fast material transfer rate (9×10^{-8} mL/s) [8, 16, 18], and contamination is avoided because there is no direct contact between laser, ribbon and substrate [18]. Spatial accuracy is better than 5 μm [16, 18], and live/dead assays reveal near 100% cell survival [15, 16, 18]. Current LBDW techniques can transfer varying numbers of cells, either single or multiple; between 0–8 cells can be dispensed per droplet and dispensed spot sizes are approximately 90 μm in diameter [16].

As laser-based direct-write techniques were not originally developed for biological material transfer, they have nevertheless a number of disadvantages including: varying transfer rate due to inhomogeneous biological layers; possibly causing genetic damage due to the UV exposure; poor reproducibility in certain cases due to variable laser interaction caused by the lack of laser absorption layer; and deposition rate limited by the laser repetition rate [18]. An added weakness of the MAPLE DW technique is its requirement of incorporating laser-absorbing matrix materials [9].

3. Inkjet printing

Inkjet printing was, until recently, used almost exclusively to print documents (e.g., typescripts prepared with a computer). With a few slight modifications, however, this technology can be

used to print bio-ink solutions containing live cells (Figure 3). Inkjet technology was first adapted for use with biological materials by Wilson and Boland in 2003 [23]; their printer was converted from an off-the-shelf inkjet document printer. Due to its low cost and high throughput, inkjet printing has become an extremely popular cell printing technique [15, 24–27].

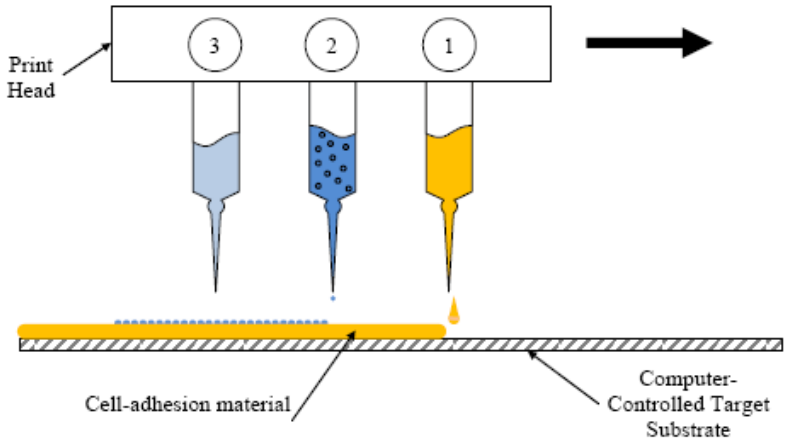

Figure 3. Simplified schematic of an inkjet system used for cell printing (adapted from [29]). Jet 2 contains the bio-ink with live cells. Jet 3 can contain any other experiment-specific bio-ink, such as proteins, other cell types, medium etc.

Inkjet printing can be defined as a technique that use the surface tension of the bio-ink itself as a valve [28]. Droplets are ejected from a nozzle by applying a pulse of pressure to the fluid (bio-ink solutions) in the supply tube, upstream of that nozzle. There are several methods of creating this pressure pulse: thermal bubble, piezoelectric, and electrostatic; they are described in the following sections. Both thermal and piezoelectric types have been modified for use as cell printers [23, 24].

3.1 Piezoelectric inkjet printing

Piezoelectric materials are crystalline materials that deform when subjected to an electric potential. There are two common types of piezoelectric material: bimorphs, which bend like a drum head, and rods, which elongate. A section of this material (either in a rod or bimorph configuration) is attached to the outer wall of the bio-ink channel just upstream of the nozzle and configured to squeeze the channel. This enables the creation of a pressure pulse that results in a droplet being ejected from the nozzle (Figure 4). The electrical pulses that energize the piezoelectric materials are typically of microsecond duration [30–32].

3.2 Thermal bubble inkjet printing

Thermal bubble inkjet operates in much the same way as piezoelectric-based printers but instead of using piezoelectric material the pressure pulse is created by a heater (Figure 5), which is composed of a thin-film resistive metallic layer, typically less than 1 micrometre thick and around 15 micrometres across each side, attached to the inner wall of the bio-ink channel just upstream of the nozzle. By passing an electrical pulse of sufficient amplitude through the heater its

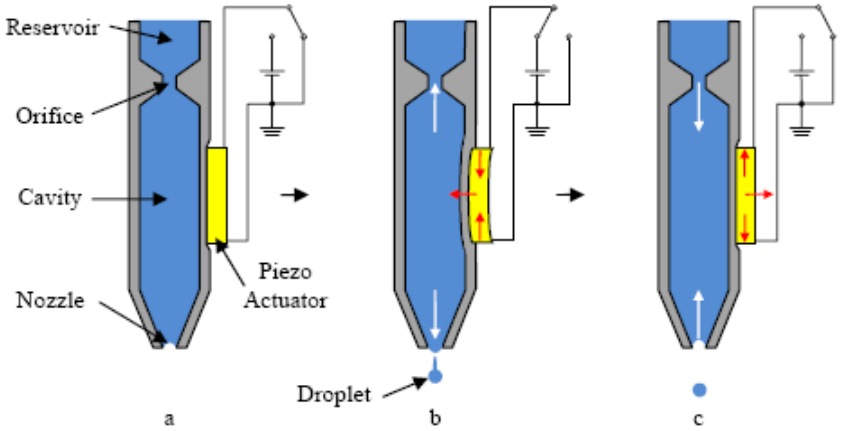

Figure 4. The process of droplet ejection in a push pull piezoelectric inkjet system: (a) initial state; (b) DC voltage applied across the piezoelectric material and a droplet is ejected (push); (c) DC voltage is removed (pull). (Adapted from [33].)

temperature rises to a point high enough to boil the bio-ink, which vaporizes within a fraction of a micrometre of the heater, forming a bubble that expands. This expansion of the bubble creates a pressure pulse that results in a droplet being ejected from the nozzle. The bubble cools and collapses after a few microseconds and the surface tension of the bio-ink meniscus at the nozzle pulls more bio-ink down from the reservoir to refill the bio-ink channel. The electrical pulses that energize the resistive materials are typically of microsecond duration [30, 31].

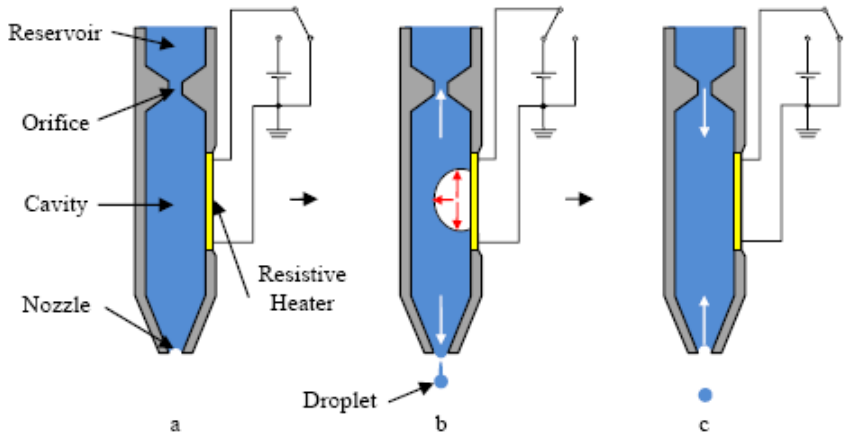

Figure 5. The process of droplet ejection in a thermal bubble inkjet system: (a) initial state; (b) DC voltage applied across the piezoelectric material and a droplet is ejected (push); (c) DC voltage is removed (pull).

3.3 Electrostatically actuated inkjet printing

SEA-JET (static-electricity actuator inkjet) printing was developed by the Seiko Epson Corporation in 1998 [34]. It was created to address the cavitation problems associated with the piezoelectric system and the large power requirements of thermal inkjet printing. The electrostatic actuator comprises a silicon pressure plate and an electrode, which are positioned

in parallel between two glass plates (Figure 6). The cavity above the pressure plate is filled with bio-ink from the reservoir.

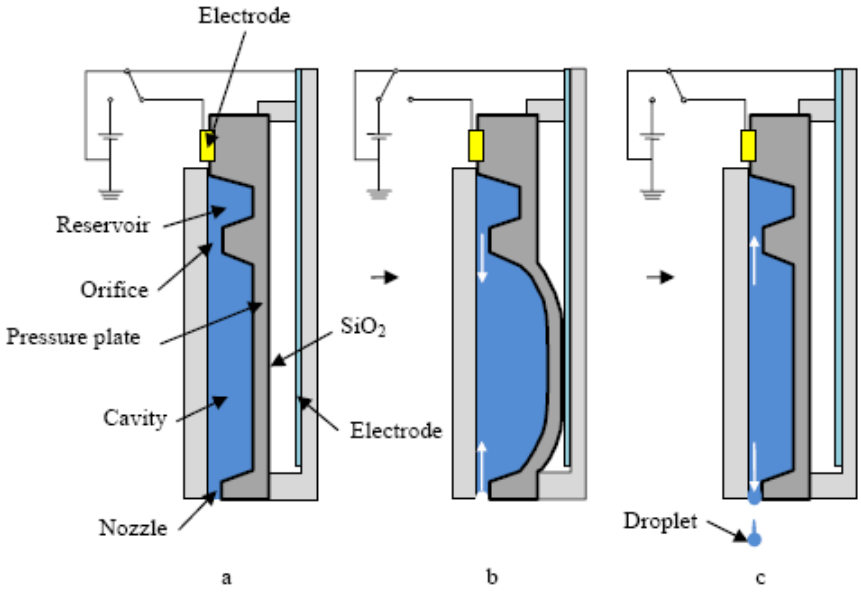

Figure 6. The process of ink ejection in an electrostatically actuated inkjet system: (a) initial state; (b) DC voltage applied between the pressure plate and the electrode (pull); (c) DC voltage is removed and a droplet is ejected (push). (Adapted from [34].)

3.4 Superfine inkjet printing

An interesting new technique for inkjet printing called *superfine inkjet printing* has recently been developed by Japan's National Institute of Advanced Industrial Science and Technology (AIST). Superfine inkjet (SIJ) printing dispenses droplets of subfemtolitre volume—only one thousandth of the volume of current inkjet devices on the market [35, 36] (Figure 7).

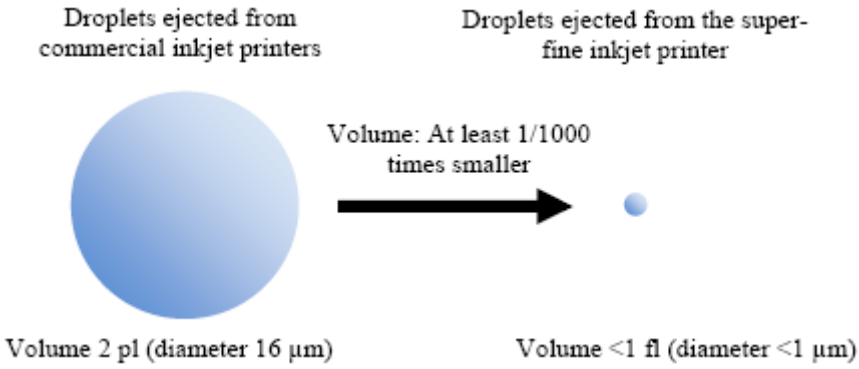

Figure 7. Comparison of standard and SIJ droplet sizes (adapted from [36]).

Unfortunately, due to the novel and proprietary nature of this technology, the majority of the technical information about it is protected, but based on what little information is available our assessment is that it would not be easily adapted to printing viable cells. SIJ printing has an accelerated drying time due to the smaller droplet sizes, which serves to counteract bulging in small features usually caused by surface tension; this could drastically reduce the viability of printed cells.

3.5 Strengths and weaknesses

Inkjet-based techniques have several advantages over others. Chief among them is the lower cost and simplicity facilitated by modifying off-the-shelf document inkjet printers. Other strong points are the ability to use several different types of cells by simply adding more nozzles [24, 37], and the intrinsically high-throughput nature of the mechanism [23].

Unfortunately inkjet-based techniques also have a number of disadvantages, the biggest of which is due to their being driven by the size of the nozzle: droplet diameters are approximately double the internal diameter of the nozzle used (which leads to larger droplet sizes compared to other techniques; hence the excitement surrounding SIJ). Nozzle clogging from cell sedimentation and aggregation is also a big problem if high cell concentrations ($>5 \times 10^6$ cells/mL) are used [27], however adding a calcium ion (Ca^{2+}) chelating agent to the bio-ink, such as EDTA (ethylenediaminetetraacetic acid), could help prevent nozzle failure and increase the bio-ink cell concentration by reducing cell aggregation [30, 38]. Other problems include a spatial accuracy of only ~50 μm (which is sufficient for cell printing but a higher spatial accuracy would be desirable), shear stress applied to the material being printed, and possible contamination [18, 24, 37, 39].

Piezoelectric-based printers have increased power requirements (12–100 W) and higher vibration frequencies (30 kHz) due to the use of high viscosity bio-ink, which is enough to break and damage cell membranes [24].

Thermal-based printers suffer from possible heating effects as temperatures can reach 300 °C or higher in some devices, which can cause many cells to die during printing [8, 24, 37].

4. Valve-based printing

Valve-based printing techniques are extremely similar to inkjet. They comprise a static pressure reservoir, a small diameter nozzle, a voltage-controlled valve, and a two-dimensional translation mechanism (the print head) to which the other components are mounted (Figure 8). The reservoir is loaded with cells that are uniformly suspended within cell culture medium. The cells are delivered to the substrate by activating the voltage-controlled valve [40, 41]. The amount of biomaterial, including cells and suspension, that is transferred can be expressed as a function of the nozzle diameter, the size of the cells, the inlet pressure and the amount of time the valve is open [42].

Strengths and weaknesses. Valve-based techniques are one of the newest additions to the collection and have the advantage of being manifestly one of the gentlest techniques for printing cells, repeatedly demonstrating extremely high final cell viability [41, 43]; this is due to the comparatively low shear stress created in this technique. Another valuable advantage is that cell numbers in the dispensed droplets are more uniform than in other techniques [43].

Other advantages include the high-throughput nature of the technique (even with a single nozzle 1000 droplets can be dispensed in less than a second [41]); as with inkjet printing it is easily expandable ("scaled out" in the terminology of microsystems and nanotechnology) and very cheap.

Just like inkjet techniques it also has nozzles and, therefore, nozzle-based problems, such as clogging and the unhelpful link between droplet diameters and nozzle diameters, remain.

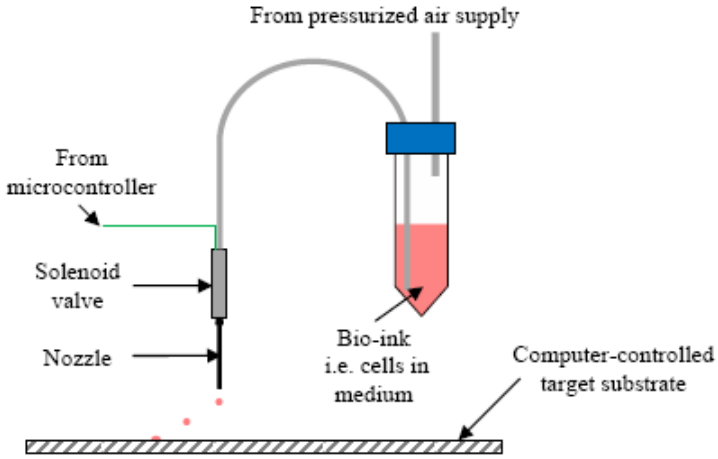

Figure 8. Schematic drawing of a valve-based cell dispensing system.

5. Optical tweezers

Optical tweezers, a well-known technique for the manipulation of nanometre and micrometre-sized particles suspended in a liquid, was pioneered in 1970 by Arthur Ashkin at Bell Labs [44]. Optical tweezers are now well established in the physical and life sciences. Forces up to 200 pN can be applied to particles with extremely small dimensions; even nanoparticles as small as 5 nm can be manipulated [45, 46], and living cells can be manoeuvred with ease. Cell throughput rates can be extremely high—in the region of $\sim 1 \times 10^6$ cells/s has been achieved [45].

A typical optical tweezer setup is shown in Figure 9. A laser beam is focused by sending it through a microscope objective; single cells can be trapped at the narrowest point of the focused laser beam, known as the beam waist. Almost all cell types can be harmlessly trapped by simply selecting a laser wavelength that is not absorbed by the cells [47].

Strengths and weaknesses. Optical tweezers have several advantages over other techniques. As this technique is noncontact, cells can be moved from one reservoir to another, with a negligible amount of extracellular medium being dragged along with them, in a few seconds. This means that there is no contamination if different types of cells are used and studies can be conducted in real time [47].

It was originally thought that the optical tweezers technique was purely noninvasive; however, studies by Liu et al. showed that cells may be heated by amounts sufficient to affect their physiological state [48].

Unfortunately this technique is only suitable for transporting cells on a single plane and the small size of the volume that can be trapped limits the number of cells that can be manipulated simultaneously [7]. Therefore, optical tweezers do not meet the demands of cell printing.

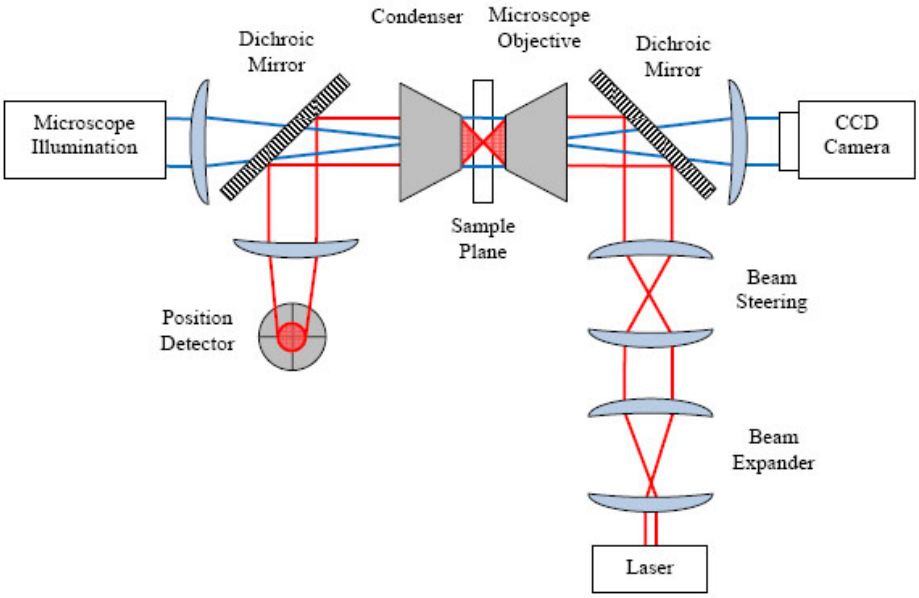

Figure 9. Simplified schematic of an optical tweezer system (adapted from [47]).

6. Electrohydrodynamic jet printing

Electrohydrodynamic jet printing (EHDJ), otherwise known as bio-electrospraying or e-jet printing, was first demonstrated in the 1980s by John Fenn at Yale University [49]. Rather than using thermal or laser energy to produce droplets, electric fields are used [50].

A typical EHDJ setup is composed of a syringe pump attached to a ~500 μm nozzle that is kept at a positive potential (0.5–0.9 kV/mm) with respect to the ground electrode above the receiving substrate positioned approximately 15 mm below the nozzle orifice (Figure 10) [51, 52]. The reservoir of the syringe is loaded with cells that are uniformly suspended to a concentration of 1×10^6–2×10^6 cells/mL [52]. In order to transfer the cells between the nozzle and the substrate a potential difference is applied between the nozzle and the ground electrode placed centrally below it.

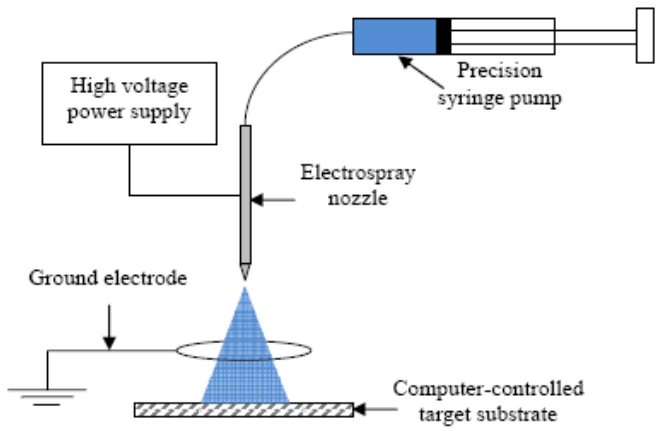

Figure 10. Simplified schematic of an electrohydrodynamic jet system used for cell printing (adapted from [52]). The cells are stored in the syringe. The jet of cells being transferred is shown as the shaded triangle below the electrospray nozzle.

Several cell types have been used, including Jurkat and mouse neuronal cells [51, 52]. Each investigation showed that the EHDJ process does not in any way damage the cells. Cells that were examined after printing exhibited growth comparable to that of control cells that had not been subjected to the process.

Strengths and weaknesses. Electrohydrodynamic jet printing presents a new advantage that none of the other techniques have yet achieved: a continuous stream of cell solution is realizable, which may be used to form cells into polymer threads that can be used as scaffolds (this process is called "electrospinning" [53]). Other strong points in its favour are 100% cell viability and the size of the droplets being independent of the nozzle size [49–52].

Unfortunately, to date no papers have been published demonstrating that electrohydrodynamic jet printing can be used to pattern cells in a controlled and reproducible manner. Another disadvantage is the wide range of droplet sizes, between tens of micrometres and millimetres in diameter, that are created during the same experiment [51, 52]. Unfortunately this means that the process, in its current form, is unsuitable for high-resolution cell printing, as position and droplet size reproducibility are an absolute requirement for the majority of cell printing applications.

7. Acoustics

The first experiments using acoustic energy to transfer liquids was carried out by Alfred Lee Loomis in 1927; he observed that upon immersing a high-power acoustic generator in an oil bath, a mound appeared on the surface that *"[erupted] oil droplets like a miniature volcano*" [54, 55].

Although there are variations among acoustic techniques, they generally operate on the same principle: an acoustic generator is placed below (or above, depending on the direction in which one wishes to dispense) the fluid to be dispensed and sends acoustic waves propagating through the fluid, which are focused at the interface between the fluid and the air, creating swellings at the focal point, which grow until they are large enough to pinch off and become droplets [55, 56].

The first version of this technique used a single focused transducer located below a microplate with multiple fluid-filled wells (Figure 11). The transducer moved from well to well and triggered the ejection of droplets by sending acoustic waves that travelled through the fluid to form a focal point, set using an acoustic lens, located just below the surface of the fluid [55, 57, 58]. This technique lacks the ability to simultaneously dispense multiple droplets unless multiple transducers are used, which would increase the already relatively high power requirements [58].

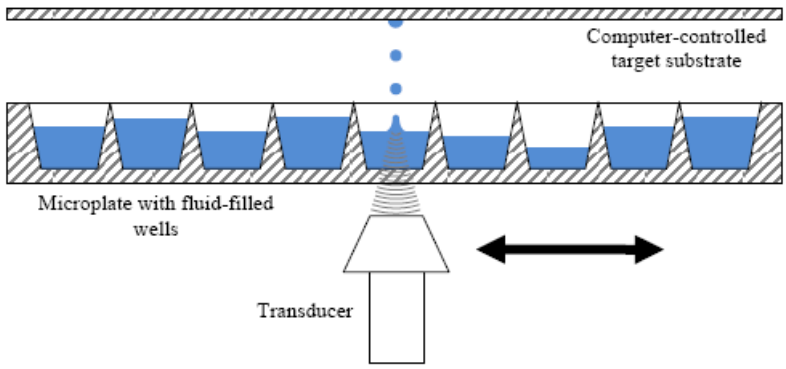

Figure 11. Simplified schematic of a classic transducer-based acoustic system (adapted from [55]).

A newer version, created by Utkan Demirci at Harvard–MIT Health Sciences and Technology and Harvard Medical School employs acoustically focused two-dimensional (2D) micromachined microdroplet ejector arrays (Figure 12). Instead of using an acoustic lens to create the focal point, it is formed by the constructive interference of surface acoustic waves leaking into the fluid medium. The substrate uniformity and ease of fabrication ensures repeatability and stable operation of the ejector array. Integrated microfluidic channels constantly replenish the fluid, keeping its level constant and allowing the array to be oriented in any direction without affecting the printing process [58, 59].

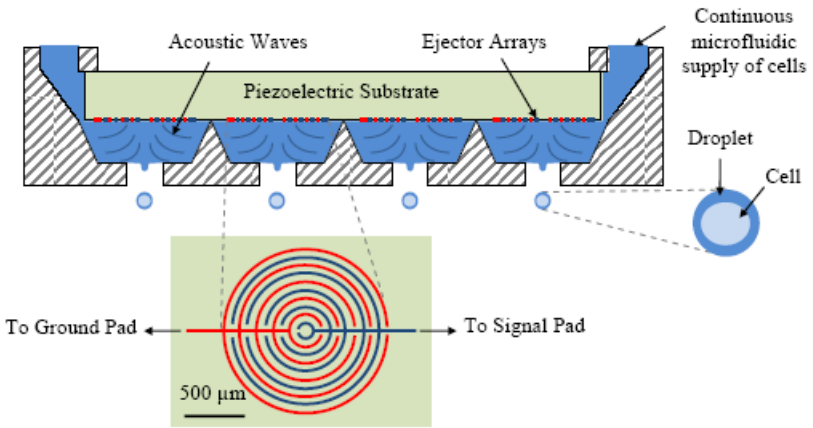

Figure 12. Simplified schematic of an interdigitated transducer-based acoustic printing system (adapted from [58, 59]).

Various types of cells have been used to test this technique including AML-12 hepatocytes, HL-1 cardiomyocytes, mouse embryonic stem cells, fibroblasts and human Raji cells encapsulated in acoustic picolitre droplets at rates varying from 1 to 10,000 droplets per second. Cell viabilities were demonstrated to be 90% or more across various cell types even at high throughput rates [58].

Strengths and weaknesses. Acoustic techniques boast many advantages over other techniques: the droplets are ejected from an open pool without requiring a nozzle, thus avoiding the problems associated with nozzles—(such as clogging, heating and high pressures), and enabling the encapsulation and ejection of single cells (or a few cells; e.g., 1–3 cells per droplet was demonstrated by Demirci & Montesano [58]) with uniform ejection directionality, high consistency, and post-ejection viability >90%. Small volume transfers (in the picolitre to nanolitre range) at low ejection velocities have been demonstrated, which reduces the chance of cross-contamination due to splashing [55–59].

A slight problem with this technique is that heat is generated by the interdigitated transducers when they trigger a droplet dispense, which raises the temperature in the fluid reservoir and could increase the evaporation rate, affect the properties of the fluid, droplet sizes and even cell viability. Fortunately the concomitant temperature rise is extremely small ($<5\times10^{-5}$ °C) and has plenty of time to dissipate before the next dispense is triggered; even at 10 kHz it only takes ~90 µs to dissipate. This temperature rise could become an issue if the ejector array were much larger or if it were operated in continuous ejection mode or at higher frequencies for a long interval [59].

8. Pyroelectric jet printing

Pyroelectric jet printing, also known as pyro-electrohydrodynamic jet printing, is another recent addition to the printing family. It is similar to the electrohydrodynamic jet printing described earlier but instead of electrodes, liquid is dispensed by heat varying the temperature of polar dielectric crystals [60].

A typical pyroelectric jet setup is shown in Figure 13. A heat source (usually a laser or even a hot soldering iron tip) is applied to a sheet of pyroelectric material (such as lithium niobate). As the pyroelectric material heats up, local electric potentials are created that initiate the electrohydrodynamic effect in the fluid on the surface of the glass. This leads to the ejection of small droplets of fluid that are printed onto an intervening substrate with nanoscale resolution [61].

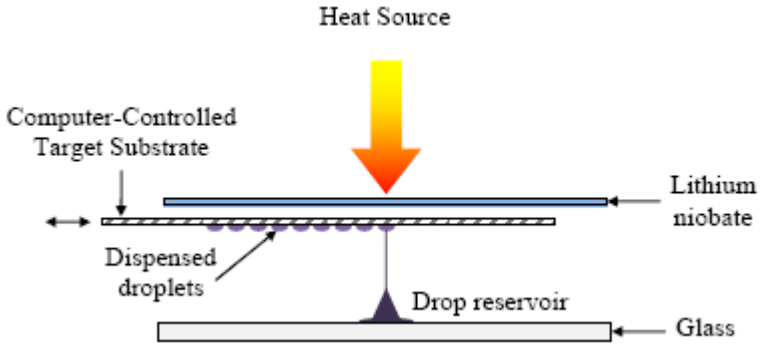

Figure 13. Simplified schematic of a pyroelectric jet printing system.

Strengths and weaknesses. Pyroelectric jet printing, although similar to electrohydrodynamic jet printing, has the advantage of no nozzles, thus avoiding the problems from which so many of the other techniques can suffer (clogging etc.). Since the electrohydrodynamic effect is triggered by a heat source, electrodes need not be used, making the system more flexible and easier to set up than EHDJ. Furthermore, attolitre droplet generation has been demonstrated with this technique [60–62]

Problems may arise due to the effect of heating on the printed droplets; the evaporation rate of the fluid could be increased and if cells are suspended within the fluid, they could be adversely affected as well.

9. Other techniques

Apart from the techniques covered in the previous sections, a number of more traditional, but still widely used, cell printing techniques exist. A selection of them is summarized in this section: including microcontact printing, microarray spotting, and photolithography.

9.1 Microcontact printing

Microcontact printing (μCP) uses a stamp created by photolithography to pattern cells onto a substrate, just like potato printing. First, a layer of photoresist is applied to a piece of silicon, which is then patterned by a photomask and UV light. Then a stamp is created by pouring

polydimethylsiloxane (PDMS) over the patterned surface and curing it at a high temperature. Finally the stamp is coated with cells in suspension and brought into contact with the substratum, transferring the cells to the substratum in the ordained pattern (Figure 14) [15, 63, 64].

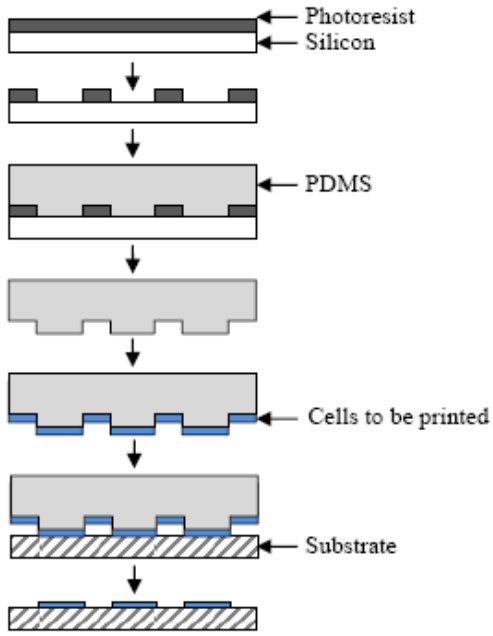

Figure 14. Schematic of the microcontact printing procedure (adapted from [65]).

Microcontact printing is a simple technique that can quickly pattern cells with a single stamping motion that can be reused to create the same pattern many times. However, a new stamp would be required for each new pattern and stamps can suffer from deformation and swelling or shrinking [15, 63–65].

A possible addition to the more modern printing techniques would be to print cell adhesion molecules using microcontact printing before delivering the cells, more definitely organizing the cells into the desired pattern [24].

9.2 Microarray spotting

Microarray spotting is similar to some of the more modern techniques in that it employs a computer controlled *xyz* motion stage to move a pen device delivering the biological material. The pen itself operates in the same way as a quill-type writing pen and is used to pick up small volumes of biological material from multiwell plates and depositing (or spotting) them in the desired location on the substratum (Figure 15) [66].

Providing that the substratum is a flat, solid surface printing is reliable and repeatable. However, if it is a soft membrane or uneven then problems can arise such as missed spots or surface indentations [66]. Microarray spotting techniques have several inherent limitations including heating linked to viscosity effects, variable volume transfer, clogging and cross-contamination (if multiple biological materials are used) [18, 66].

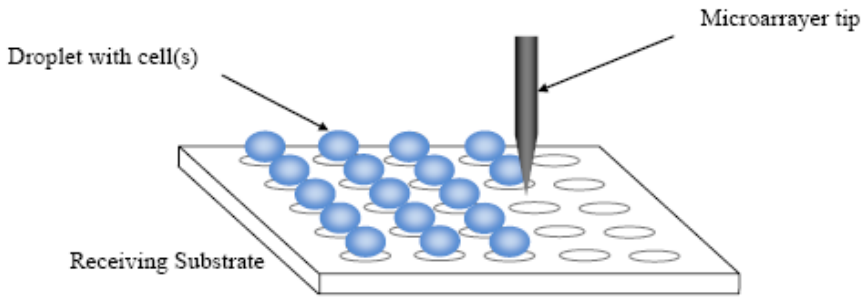

Figure 15. Schematic of the microarray spotting technique (adapted from [67]).

9.3 Photolithography

Photolithography, also known as optical or UV lithography is a microfabrication technique that uses light to remove parts of a thin film or substrate. The desired pattern is transferred using light passing through a photomask onto a light-sensitive chemical photoresist on the substrate [68, 69]. A series of chemicals are applied that etch the exposure pattern onto the substrate. Photolithography is used in the semiconductor industry to create complex integrated circuits.

With slight modification, the photolithographic process can be used to pattern cell deposits. By depositing cell attachment factors (CAF) onto a substrate previously coated with agar (which retards cell adhesion) and imposing a pattern onto the CAF using photolithography the resulting surface will only permit cells to grow in the desired pattern (Figure 16). This method has been successfully used to pattern fibroblasts, cardiomyocytes and HeLa cells with a spatial resolution of ±3 μm [65, 68].

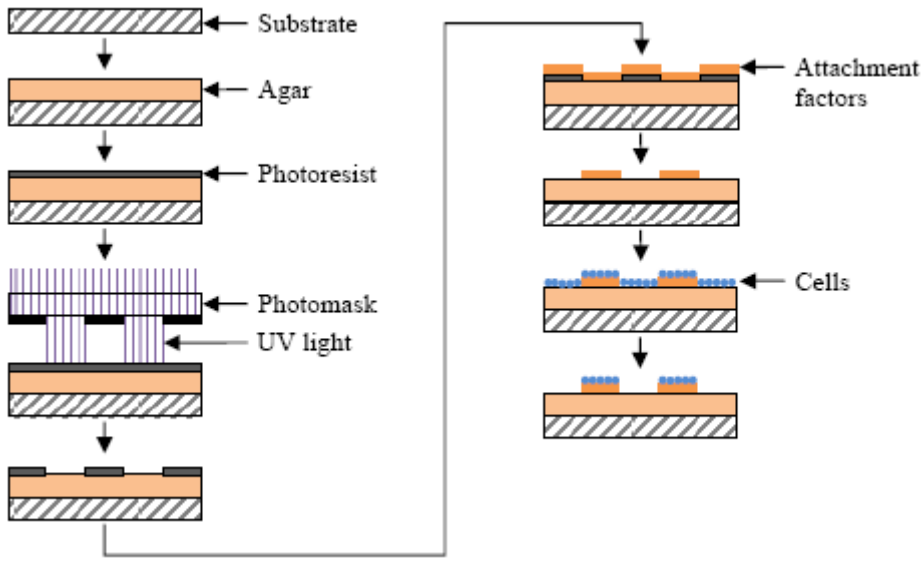

Figure 16. Schematic of the photolithographic cell patterning technique.

Photolithography is an extremely useful microfabrication technique; so much so that it is often used to supplement some of the other techniques described in this review (e.g., making patterned substrates onto which cells are printed [70] or stamps with which to print cells [65]). Unfortunately it is also an extremely expensive process (which only makes it viable for mass production) and it is unable to pattern nonplanar surfaces [65].

10. Applications of biological cell printing

During this investigation into the techniques already used or potentially useful for biological cell printing it became apparent that there is a wide range of actual and promising applications. They range from *in vitro* drug screening to organ printing, including tissue engineering, stem cell and cancer research.

10. 1 Microcantilever-based biosensor printing

A biosensor is a chemical or biochemical sensor that uses a biological molecule, virus or cell to selectively capture the analyte of interest, following which its presence is detected by a transducer. Transducers may be electrical, optical etc. In this section we focus on a mechanical transducer—the microcantilever. However, the principles we describe apply to other transducer types as well. In the direct application of cell printing, the technique is used to apply cells to the transducer surface, where they constitute the capture layer. The coated transducer responds to anything that changes the morphological state of the cells (including its viscoelasticity). The transducer can also be coated with a cell adhesion layer and used as a sensor for the cells themselves.

Microcantilevers have been used to study cells, bacteria and viruses and they provide an inexpensive and quick detection system [71]. Microscale cantilever-based resonators have demonstrated their high sensitivity, in the range of femtograms, as biosensors [72]. Antibodies specific to certain cells or bacteria can be coated on the surface of the cantilever, which results in an extremely high selectivity to certain strains of pathogens. The adhesion of cells onto the surface of the microcantilever alters its resonant frequency; by measuring this change the presence of different cells (including bacteria and viruses) can be determined. Gfeller et al. used this principle of resonant frequency-based mass detection to monitor the growth of *Escherichia coli* bacteria; they discovered that this method has sufficient sensitivity to detect a single bacterial cell. Moreover, the sensors were able to detect *E. coli* in less than 1 hour compared to the 24 hours or more required by conventional cell culturing techniques [73]. This could be extremely valuable to the food industry where rapid detection of bacterial growth is crucial. Microcantilevers can also be operated in static mode: Ghayal et al. coated one side of a cantilever array with short peptide ligands; when *Bacillus subtilis* spores were introduced they adhered to the ligands and a 40 nm deflexion was measured (spore presence was confirmed using dark-field microscopy) [74].

Microcantilevers can also be used for selective immobilization and rapid detection of different cells such as *Saccharomyces cerevisiae* and *Aspergillus niger*, demonstrated by Nugaeva et al. [75]. They showed that precoating the cantilevers with concanavalin A, fibronectin or immunoglobin G resulted in differential adhesion of the different cells. Gunter et al.

fabricated piezoresistive microcantilever beams able to detect single vaccinia virus particles (which have a mass of only 9.5 femtograms) producing distinct signals from virus adsorption to a cantilever surface coating of vaccinia antibodies [76].

10. 2 Tissue engineering

From the very first article that was published investigating cell printing, tissue engineering was identified as a major application for the new technology. Cell printers that are only able to create two-dimensional cellular constructs are useful for some applications, such as printing skin [12], but a number of studies have shown that many types of cells require a three-dimensional (3D) structure in order to function properly. For example, Dunn et al. showed that hepatocytes cultured as a monolayer lost many of their liver-specific functions after a few days, but those cultured with layers of collagen gel in a sandwich configuration were able to retain their liver-specific functions for weeks [77]. Therefore, if more complex structures such as organs were to be printed, the cell printer would need the ability to transfer mesoscopic patterns of viable cells and appropriate biomacromolecules providing an extracellular matrix into well defined three-dimensional arrays that closely mimic real tissue structure.

10. 3 Cell sorting and research

One of the most important requirements for studying the components of any biological system, either molecules in a cell or cells in an organ, is the ability to procure pure populations of different types of living cells from the biological system being studied [78]. These isolated components can then be characterized before being recombined under controlled conditions.

The ability to create precise *in vitro* cultures of cells is essential for replicating the *in vivo* microenvironment. For example, a culture of cancer cells can help us to gain further understanding of the influence of spatial and geometric locations on cancer induction, proliferation and metastasis [15]. Cell-to-cell communication between healthy normal cells and carcinomas could evidently also be studied in cultures, making it much easier to infer causal relations given the highly defined nature of a printed culture.

Likewise for drug development, a cell-based model that emulates *in vitro* behaviour offers obvious advantages over traditional drug testing techniques—saving time, money and increasing predictability [79]. The use of model cell cultures is also aligned with current ethical norms in biomedical research that endeavour to minimize the use of animals.

Cell printing techniques have amply demonstrated their ability to place cells into precise, preprogrammed patterns—examples of such control are Guillotin et al. with their Olympics logo printed using the LIFT technique [19] or the Heriot-Watt University logo printed using our own dual valve-based printer (described previously) [80] (Figure 17).

11. Summary and conclusion

Table 2 compares the capabilities of the different techniques for printing viable biological cells.

There are many different approaches to cell printing, but simply described they are, generically, rapid transfer techniques that are easily customized in terms of cell types, printed patterns and applications. Many of the techniques utilize CAD/CAM technology (be that

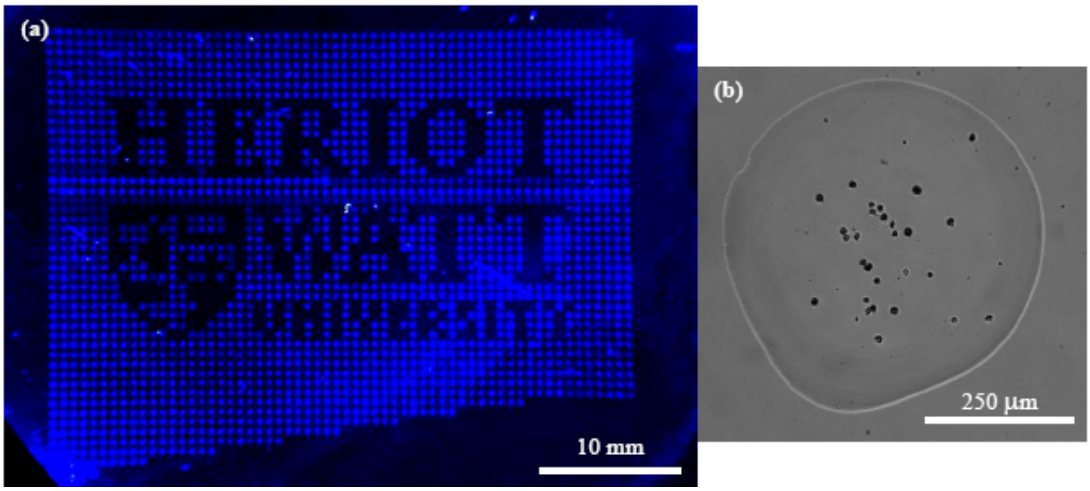

Figure 17. (a) fluorescence image of an array of Human Embryonic Stem cell droplets printed in the form of the Heriot–Watt University logo; (b) optical image of a single droplet showing the RC-10 cells (from [80]).

Table 2. A comparison of the demonstrated capabilities of the different techniques examined in this review.

Technique	Spot size or resolution (µm)	Deposition speed	Maximum cell throughput (cells/s)	Cell viability (%)	Advantages/*limitations*
Laser-based	10–120	9×10^{-8} ml/s	1×10^{4}	95–100	Nozzle-free, *expensive, radiation*
Inkjet	50	5×10^{3} drops/s	850	75–90	Expandable, multi-ink, cheap, *nozzle-based, heat/viscosity*
Valve-based	~10	1×10^{3} drops/s	1×10^{5}	>90	Gentle, expandable, multi-ink, *nozzle-based*
Optical Tweezers	5×10^{-3}	1×10^{6} drops/s	1×10^{6}	100	Single plane transport only
ElectroHydro-Dynamic Jet	50–1000	0.01 ml/s	2×10^{4}	100	Continuous streams, *non-fixed droplet sizes*
Acoustic	~12	1×10^{4} drops/s	3×10^{4}	>90	Nozzle-free, multi-ink
Pyroelectric Jet	30–70	400 pl/s	No Data	No Data	Nozzle-free, *heat*

software or machinery) and have achieved, or are very close to, single cell resolution. Cells can be deposited onto a homogeneous growth surface to ensure cellular proliferation controlled by normal cell–cell interactions. Precise patterns of cells can be deposited to form cocultures and multicultures. Three-dimensional printing is possible via depositing cells layer-by-layer, either by repeated deposition of cells onto a single spot or by the addition of matrix layers. The majority of cell printing techniques are capable of transferring cells quickly and reliably while keeping a high proportion of them viable (alive). Doubtless new techniques will continue to emerge as the field develops.

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