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Inkjet printing of viable mammalian cells

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Abstract

The purpose of this study was to explore the use of a commercial thermal printer to deposit Chinese Hamster Ovary (CHO) and embryonic motoneuron cells into pre-defined patterns. These experiments were undertaken to verify the biocompatibility of thermal inkjet printing of mammalian cells and the ability to assemble them into viable constructs. Using a modified Hewlett Packard (HP) 550C computer printer and an HP 51626a ink cartridge, CHO cells and rat embryonic motoneurons were suspended separately in a concentrated phosphate buffered saline solution ($3\times$). The cells were subsequently printed as a kind of "ink" onto several "bio-papers" made from soy agar and collagen gel. The appearance of the CHO cells and motoneurons on the bio-papers indicated an healthy cell morphology. Furthermore, the analyses of the CHO cell viability showed that less than 8% of the cells were lysed during printing. These data indicate that mammalian cells can be effectively delivered by a modified thermal inkjet printer onto biological substrates and that they retain their ability to function. The computer-aided inkjet printing of viable mammalian cells holds potential for creating living tissue analogs, and may eventually lead to the construction of engineered human organs.
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1. Introduction

This work has demonstrated that viable mammalian cells be delivered in pre-determined patterns using a modified inkjet printer. This new ability has implications for any application that requires spatially registered cellular engineering, primarily in the field of tissue engineering, which is a rapidly expanding approach to address the shortage of organs for transplantation [1]. In the most general sense, tissue engineering seeks to fabricate viable replacement parts for the body [2]. Although still in its infancy, much progress in tissue engineering has been made in areas relevant to the development of novel biomaterials [3], the design of bioreactors for dynamic in vitro culture systems [4], and the use of a wide variety of cell sources, including appropriate multi-potent stem cells [5]. Advancements in tissue engineering have permitted the creation of functional tissue substitutes available to patients for clinical applications, including engineered stomachs [6], esophagus [7], spinal cord [8], and progress towards complete tissue engineered organs [9]. To further the construction of complex tissues, or even entire organs, however, there are still significant technical challenges to overcome. Among them, a very important problem is subtly combining and orchestrating cells, growth factors and scaffolds into an architecture that will allow their unfettered interaction, especially where distinct cell types are required in anatomically exact locations to attain biological function [10]. Inkjet printing technology offers a possible solution to this complex problem.

Inkjet printing, initially from the field of electronics and mechanics [11], has recently been extended to bioengineering applications [12]. With the obvious advantages of being inexpensive as well as high throughput, commercial thermal inkjet printers have been modified to print biomolecules onto target substrates with little or no reduction of their bioactivities, resulting in the creation of DNA chips [13], protein arrays [14] and cell patterns [15]. In particular, our recent success in printing viable bacteria directly using an off-the-self thermal inkjet printer [16] significantly expands the capabilities of inkjet printing in the direction of creating living tissue substitutes. By means

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of computer-assisted deposition, viable cells can be delivered to precise target positions on scaffold materials. Furthermore, using different cell types as different bio-inks, which are then delivered to exact positions to mimic tissue structures of the original tissue, can be envisioned by using multiple nozzles. Thus, the printing of dissociated human or animal cells onto specific patterns, and their subsequent fusion, may allow the development of replacement tissue or even whole organ substitutes [17]. Compared to bacteria, however, animal cells are generally more sensitive to heat and mechanical stress, both of which often occur in the cartridge of a printer. Whether animal cells could be effectively printed using the modified thermal printer still needed to be demonstrated to realize this ambitious goal.

In the present study, suspensions of Chinese Hamster Ovary (CHO) and embryonic rat motoneurons were printed directly using a modified Hewlett Packard (HP) 550C printer. This thermal printer was chosen over the piezo-electric technology because our previous experience in adapting these commercial inkjet printers for biomaterial printing [15–19]. Soy agar and collagen hydrogels were used as the culture substrate or "bio-paper" to prevent the cells from drying and to provide nutrients for cell growth. The cell viability during the printing was evaluated by a combined lactate dehydrogenase (LDH) and tetrazolium compound assay (MTS).

2. Materials and methods

2.1. Cell preparation and the cell print suspension ("bio-ink")

Two different cell types were used for the inkjet printing experiments. One was a cell line of Chinese Hamster Ovary (CHO-B2) cells. These cells had been transfected to express green fluorescent protein and $\alpha5\beta1$ integrin which causes them to form compact, spherical aggregates [20]. Cells with green fluorescence are easy to observe for the printed patterns and other applications. The other cells used in this study were primary embryonic motoneurons obtained from ventral cords of day 14 embryos from pregnant Sprague–Dawley rats as previously described [21].

Cell pellets were collected by centrifugation (1000 rpm/2 min) in a conical tube. After aspirating the supernatant, they were resuspended in 0.5 ml of 3 × Dulbecco's phosphate buffered saline solution (DPBS) (Sigma Chemical Inc., St. Louis, MO) to obtain cell print suspensions (like "inks" for the printers). The concentration of the CHO cell's print suspension was about 5,000,000 cells/ml, and the primary motoneuron concentration was around 2,000,000 cells/ml.

2.2. Bio-paper preparation

The two hydrogel-based substrates were prepared as the print substrates (or bio-papers). One of the bio-papers used was soy agar gel, and the other was a collagen gel. Both were coated on glass coverslips ($22 \times 22\,mm^2$). The soy agar gel was made from Trypticase® soy agar solution (Becton Dickinson & Co., Cockeysville, MD) by dissolving 1.5 g of powdered soy agar in 50 ml of nanopure water. After autoclaving for 15 min, 50 ml of a 2 × solution of Dulbecco's modified Eagles medium (DMEM) (Sigma) was added to the soy agar solution while it still was relatively hot (about 45°C). After mixing thoroughly, 2 ml aliquots of the mixture were dispensed onto each coverslip, which was in 35 mm dishes. The soy agar gel formed on the coverslips after cooling to room temperature. The collagen gel bio-paper was produced from rat-tail Type I collagen (BD Biosciences, Billerica, MA), which was received at a concentration of 3.67 mg/ml in acetic acid. The collagen solution obtained a final concentration of 1.0 mg/ml by adding chilled PBS (2 ×). After adjusting the pH to about 7.4, 1.5 ml aliquots of the mixture were dispersed onto coverslips and plated in an incubator for 3–5 h. Once the gel formed, the bio-paper of collagen was ready for use.

2.3. Cell printing and cell culture

A modified HP Desktop printer (HP 550C) and a modified HP 51626a ink cartridge were used to print the CHO cells and motoneurons directly into specific patterns. The printer modifications are described previously [18]. For these experiments, after emptying the ink, the cartridge was rinsed thoroughly with 100% ethanol and sterile water. Previously, this procedure was proven to be enough to create uncontaminated bacterial cell cultures [16]. A pattern that consisted of rows of circles was designed using Microsoft PowerPoint software (Microsoft, Redmond, WA) to program the printer.

To form the patterns, the cell print suspension was shaken forcefully, and then a small portion (0.3–0.5 ml) of the suspension was loaded into the ink-containing chamber on the bottom of the cartridge. The hydrogel-based bio-papers were placed onto the print basement of the modified printer. The cells were delivered onto the bio-paper following the designed pattern and the cells were printed several consecutive times (2–8 ×) at the same position to ensure a high cell number on the circular patterns. After printing, the bio-papers with the printed cells were placed into dishes and moved to the incubator, which was maintained at 37°C, 5% CO_2, 95% air and 99% relative humidity. After 1–2 h incubation, a small amount of culture medium was carefully added to the dishes to avoid disturbing the

printed cell patterns and the level of the culture medium was maintained to just below the top layer of the gel. DMEM medium, supplemented with 10% fetal bovine serum (Sigma), 0.15% (w/v) sodium bicarbonate and 1% antibiotic/antimycotic (Sigma Chemical Inc.) solution was used to feed the CHO cells. The medium was changed every 1–2 days and the growth of the cells was monitored daily via light and epifluorescent microscopy. The culture medium for the primary motoneurons was Neurobasal (Gibco-BRL) supplemented with B27 (2% v/v) (Invitrogen, Carlsbad, CA), L-glutamine (0.5 mM) and 2-mercaptoethanol (25 μM). The culture medium was changed every four days and L-glutamate (25 μM) was added to the medium during the first four days of culture.

2.4. Cell viability

The amount of LDH, a stable cytosolic enzyme that is released upon cell lysis, was measured quantitatively using a commercial calorimetric assay (Cytotox 96, Promega Corp., Madison, WI). Per the manufacturer's protocol, 500 μl of reagent was added per ml of media into culture dishes containing the printed samples. As a control, a known number of cells (0, 1000, 5000, 10000, and 20,000, as determined by triplicate hemocytometer counts) were lysed using 9% (v/v) Triton® X-100; the other controls were samples coated with the gel but without cells. The absorbance at 490 nm of the solution was measured after 30 min of incubation at room temperature in the dark. The amount of live cells was determined by a tetrazolium compound assay (MTS; CellTiter 96 Promega Corp., Madison, WI). Per the manufacturer's protocol, 500 μl of reagent was added per ml of media into the culture dishes previously used for the LDH assay. The absorbances for a known amount of cells as well as for the pure gel samples were used as controls. The samples and controls were incubated for 60 min at 37°C in the dark. The total number of cells was calculated as the sum of the lysed and metabolically active cells, and the percentage of cells lysed by printing was estimated as the ratio of the lysed cells to the total number of cells.

3. Results

3.1. Cell printing and cell pattern

The CHO cells were observed to still emit green fluorescence by epifluorescent microscopy, which strongly suggested that the CHO cells were still living after passing through the nozzle of the cartridge. During the initial 5 days of culture, the printed cells did not from a complete ring because the initial plating was at low density, which obscured the ring pattern. After 6 days, the cells gradually occupied the positions along the ring pattern to form parts of the cell ring, indicating the retention of the ability to proliferate and the long-term viability of the technique. At day 7, the complete ring of CHO cells was observed on the soy agar bio-paper (Fig. 1A). The cells continued to proliferate until a complete circle was formed by day 25. Since CHO cells are not contact inhibited, eventually the entire gel became populated with cells.

A printed neuronal ring was obvious after 1 day in culture, as shown in Fig. 2A. Many rounded cells were seen, indicative of non-differentiated or dead cells. Most of the cells that survived the passage through the nozzle grew processes. Few differentiated neurons with processes were obvious at the pattern at day 2 in culture, but after 5 days, many neurons began to differentiate and developed processes, exhibiting a polarized morphology (Figs. 2C and D).

3.2. CHO cell viability analyses

Table 1 shows the percentages of lysed CHO cells after the bio-ink formulation and the printing process. On average, a very small amount of cells was lysed during the printing process, $3.3 \pm 3.7\%$ ($n = 4$); but in all cases the number of lysed cells was below 10%. The total number of printed cells ranged from 44,000 to 154,000 in these experiments. The number depended on the particular pattern and the number of repetitive nozzle firings. These numbers are well below the manual controls, which may explain the high standard deviations. The bio-ink formulation lysed on average $15 \pm 0.7\%$ of the CHO cells.

4. Discussion

4.1. Cell printing and viability

By replacing the regular print ink, the cell suspensions can be considered "bio-ink" that could be delivered using a commercial, thermal-based inkjet printer. Our previous study proved the ability to print viable bacteria directly by the inkjet printing method [16]. However, it had not been reported whether viable mammalian cells, the cells needed for building engineered tissues and organs, could be used as a bio-ink and printed directly by a commercial inkjet printer. Since the physiological properties of mammalian cells strongly depend on the culture conditions and they are much more sensitive to heat and mechanical stress, there was a major concern that the cells could be damaged or lysed by the conditions present during thermal printing. The temperature in the nozzle of the cartridge can be 300°C or higher [22]. The present study indicates, however, that viable CHO cells and primary motoneurons can be

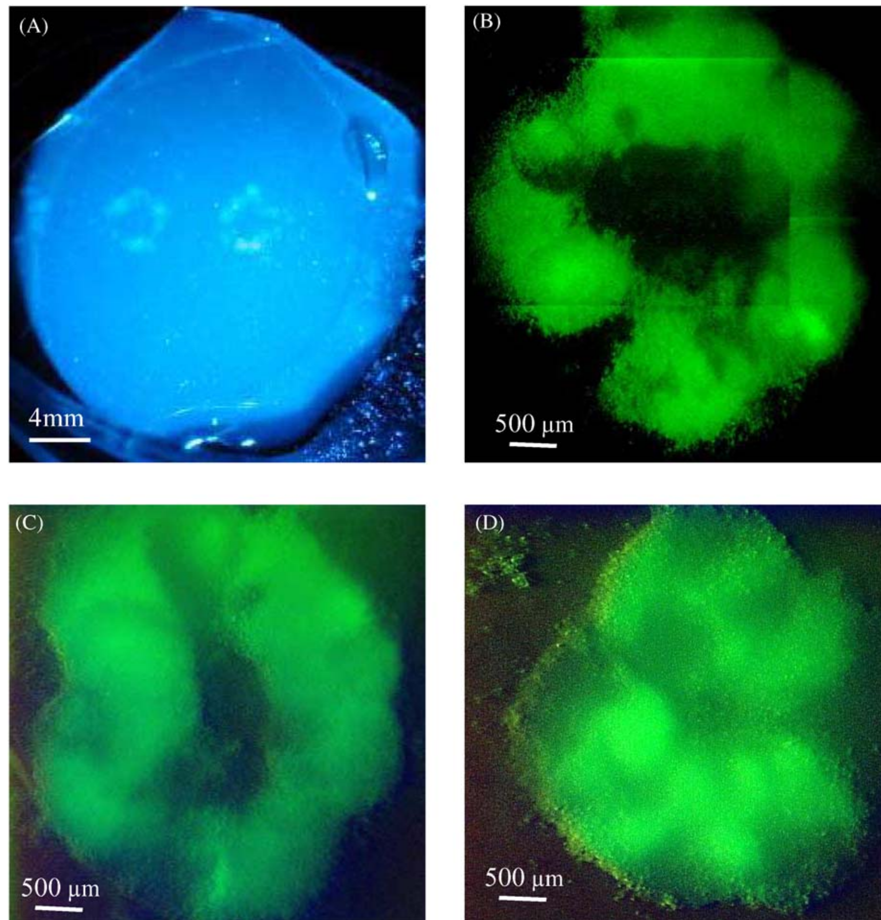

Fig. 1. Chinese hamster ovary cells visualized under a fluorescent microscope. The pattern as seen on the agar gel bio-paper at 7 days of culture (A); the pattern after 15 days of culture (B); the pattern after 20 days of culture (C), and the pattern after 25 days of culture (D). Since CHO cells are not contact-inhibited, the cells eventually grew everywhere in the gel bio-paper. Magnifications are as follows: $2\times$ (A), and $12\times$ (B, C, and D).

delivered successfully by using a modified inkjet HP printer, and most of these cells ($>90\%$) were not lysed during printing as shown in Table 1.

These data confirm our previous analysis, that cells can survive thermal printing under low power conditions [16]. The HP inkjet printer technology is based on vaporizing a micrometer-sized layer of liquid in contact with a thin film resistor. As the timescales involved in the drop ejection process are small, there is not enough time for heat to diffuse into the bulk liquid. While the surface in contact with the liquid can peak at 250–350°C, the bulk liquid does not rise more than a couple (approximately 4–10°C) degrees above ambient [23]. This situation could change however, depending on the liquid thermophysical properties of the ink. Although mammalian cells are more sensitive to high heat and strong mechanical stress than bacteria, their volume is also relatively small compared to the total volume of the printed droplet. The fact that these mammalian cells are viable, can proliferate and differentiate indicates that damage from the heat and mechanical stress during the very short timescale of printing is avoided. Moreover, the $3\times$ PBS used for the cell print suspensions could have caused a further decrease in cell volume, because they are expected to shrink by osmosis in this hypertonic solution, effectively preventing clogging of the nozzle during cell printing. This would come at the expense of higher cell death in the ink formulation. However, once deposited on the gel, the CHO cells returned to their normal shape and morphology to a great extent. The survival of motoneurons was indicated by the outgrowth of their processes and the establishment of polarized morphologies (Figs. 2B–D). However, the lengths of the processes were shorter than that observed in the standard 2D culture [21]. One of the possible reasons could be related to the use of the 3D collagen gel based bio-paper. Prior studies have found that increasing collagen concentration will decrease the neurite outgrowth [24].

Inkjet printers based on piezo-electric technology may also be modified for cell printing as they have many applications in ceramics and polymer printing. However, there are some challenges in adapting commercial piezo-printers for printing mammalian cells. For many

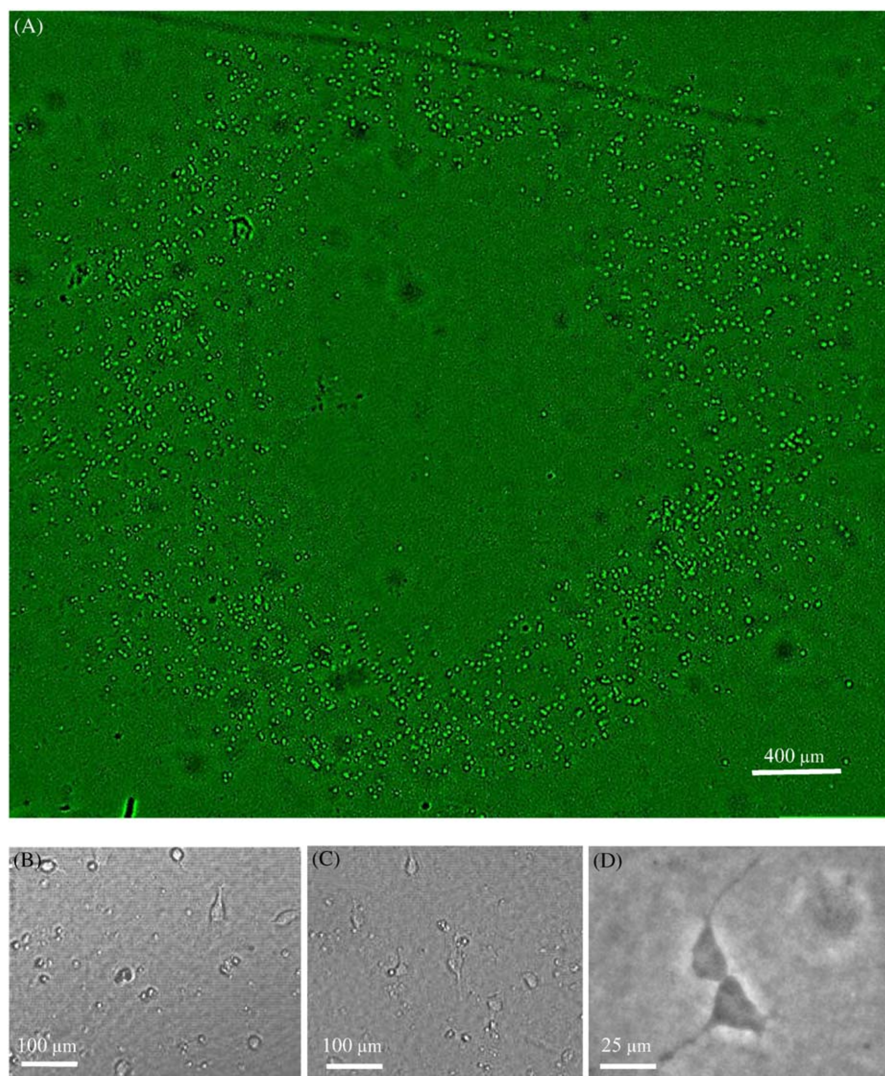

Fig. 2. Primary motoneurons were delivered by direct inkjet printing in the pre-designed ring patterns as indicated as at day 1 utilizing bright field miscopy (A). Motoneurons on the ring patterns with developing processes at day 2 observed with bright field miscopy. A few neurons are extending their processes (B). At day 3 many of the motoneurons exhibited a characteristic neuronal morphology as seen with bright field miscopy (C). At day 7 the processes of two neurons have grown to about 40 microns in length (D). Magnifications are as follows: 40 × (A), 100 × (B and C), and 400 × (D).

Table 1
Bio-ink formulation and printing cytotoxicity as indicated by the combined lactate dehydrogenase (LDH) and tetrazolium compound assay (MTS)

	Num. of lysed cells	Num. of live cells	Cells lysed by bio-ink formulation	Cells lysed by printing
Controls ($n = 3$)	60,000 ± 2000	340,000 ± 2000	15.00 ± 0.65%	0 ± 0.65%
Printed #1	26,060	128,200	0.1689	0.0159
Printed #2	7501	36,626	0.17	0.017
Printed #3	16,186	82,409	0.1642	0.0112
Printed #4	23,293	73,252	0.2413	0.0882
Average ($n = 4$)	18,259	80,120	0.1861	0.0331
SD ($n = 4$)	8290	37,661	0.0369	0.0369

reasons, including minimizing ink leakage and preventing formation of mist, commercial piezo-printers use a more viscous ink. This helps eliminate the need for complex fluid gates between the ink cartridge and printhead to prevent the ink from backflowing. However, this comes at the expense of requiring more power and higher vibration frequencies, both of which can break and damage the cell membranes. Typical

commercial piezo-printers use frequencies up to 30 kHz and power sources ranging from 12 to 100 W. This becomes a problem because vibrating frequencies ranging from 15 to 25 kHz and power sources from 10 to 375 W are often used to disrupt cell membranes [25]. Adapting piezo-printers for less viscous ink to lower the frequency and power would be challenging, since ink leakage and mist formation during printing could obscure the pattern. While these problems may be overcome, this study focused on adaptation of commercial printers without significant redesigning.

4.2. Bio-paper

Cell printing is different from printing of bioactive molecules such as DNA or proteins. While the latter are deposited onto dry substrates, and thus dry out quickly after deposition, the former require suitable materials to prevent drying of the cells. Viability of the cells is strongly affected by the drying process of the ink droplets after printing. We have earlier pointed to hydrogels as a possible solution to the drying problem [19] as they can provide the cells with water as well as nutrients and mechanical support during this time. Two different hydrogels were used as substrates in this study. For the fast, anchorage independent proliferating CHO cells, a gel that limits the migration was needed; thus soy agar hydrogels were used. With no or limited cell motility, CHO cells survived and multiplied only around the positions where they were printed, thus allowing the CHO cell pattern to fuse into to a clear ring rather than a disk. For the anchorage dependent motoneurons, a gel that promoted differentiation was needed. Soy agar gel could not be used as a substrate for neuronal printing and pattering as neurons are thought to be difficult to differentiate on the soy agar gels [26]. Since collagen had been shown to promote neuron differentiation [27], the collagen gel-based bio-paper was used for the neuronal printing. These two gels were chosen because they ensured cell survival, division and differentiation for cells representing either less or fully differentiated phenotypes.

The behaviors of cells on or within the bio-paper are directed by cell–material interactions [3,28]. Recently, it was demonstrated that the self-assembling and/or fusion of CHO aggregates embedded into 3D hydrogels depended greatly on the magnitude of cell–cell and cell–matrix interactions in the 3D systems [17,29]. It was found, depending on the amount of cell adhesive ligands in the gel, that CHO aggregates either fused into a torodial 3D structure (when using RGD containing NeuroGel or 1.0 mg/ml collagen gel) or dispersed into the surrounding matrix (when using 1.7 mg/ml collagen gel). These findings could lead to the creation of functional bio-papers, especially when combined with our previous report of collagen printing [15]. Collagen, RGD, or other cell-adhesion molecules could be printed first using a separate nozzle before delivering the cells as a second step to achieve this goal. Such multiprocessing would combine functional bio-papers for controlled cell–gel interactions, with automated delivery of cells, resulting in self-organization of cells or cell aggregates into metastable tissue structures of the desired shape.

4.3. The cell printer and biocompatibility

Few tools have been applied effectively to date to directly deliver viable cells onto pre-defined patterns for the purposes of cell patterning and tissue construction [30]. The laser-directed cell writing system is one of the few examples of direct cell placement [31]. Limited throughput and the difficulties in extending this technology into 3D constructs, however, have limited its potential application into product development or tissue construction. By definition a non-contact technique [32], inkjet printers combine micro-electronic, micro-mechanic, and computer elements into a simple, yet efficient platform, offering a new a paradigm for the precise delivery of bio-ink droplets onto exact positions on a substrate. Inspired by traditional inkjet technology, bio-printers have been developed to perform the computer-assisted deposition of natural materials, including bioactive molecules [18], biomolecules [13–15] and cells [16,19]. Direct patterning of viable mammalian cells onto hydrogel-bases papers using our cell printer could be a significant advance in living biomaterial processing and shows progress in our understanding and manipulation of natural systems. The cell printer juxtaposes electronic, mechanic, and biomaterial platforms, and is compatible with all key elements required for precise arrangement of viable cell in tissue engineering, as well as those of the control of biological molecules [13]. Moreover, this cell printer provides a useful tool for building 3D living tissues or even organ substitutes, especially when used in combination with the ability to print other cell-friendly materials or scaffolds adjacent to viable cells, as shown by our previous work to rapidly fabricate cell patterns [15]. While this initial study was mainly concerned with the survivability of mammalian cells, future studies will need to optimize bio-ink and bio-paper formulations for this technology to be successfully used in the field of tissue engineering.

5. Conclusions

The CHO and motoneuron cells were successfully printed through the nozzles of a thermal inkjet printer. The printed cells were deposited onto gel-based bio-papers with different adhesive properties to form pre-defined patterns. The analysis of the CHO cell

viability showed that more than 90% of cells were not damaged during the nozzle firing. An exciting combination of off-the-shelf inkjet printing and viable biomaterials was demonstrated for cell patterning and now could be adapted to the application of tissue engineering. With the obvious advantages of high throughput and flexibility, this bottom-up technology offers researchers a cost-effective tool to rapidly fabricate cell patterns and tissue-like structures.

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