Viability and electrophysiology of neural cell structures generated by the inkjet printing method

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Abstract

Complex cellular patterns and structures were created by automated and direct inkjet printing of primary embryonic hippocampal and cortical neurons. Immunostaining analysis and whole-cell patch-clamp recordings showed that embryonic hippocampal and cortical neurons maintained basic cellular properties and functions, including normal, healthy neuronal phenotypes and electrophysiological characteristics, after being printed through thermal inkjet nozzles. In addition, in this study a new method was developed to create 3D cellular structures: sheets of neural cells were layered on each other (layer-by-layer process) by alternate inkjet printing of NT2 cells and fibrin gels. These results and findings, taken together, show that inkjet printing is rapidly evolving into a digital fabrication method to build functional neural structures that may eventually find applications in neural tissue engineering.

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Keywords: Inkjet printing; Neural cell structure; Tissue engineering; 3D constructs

1. Introduction

Rapid fabrication of tissue or organ analogs with well-defined structures and functions is a main challenge in tissue engineering/regenerative medicine [1]. In particular, it is thought that tissue-engineered neural cell-based structures may present an alternative strategy to alleviate serious clinical conditions of the nervous system, such as brain and spinal cord injuries and neurodegenerative diseases, in which functional neural cells are often lost or degenerated within the nervous system [2]. Since most of the neuronal cells, especially those in the central nervous system, have a very limited ability to regenerate, one promising therapeutic strategy is the implantation of healthy, in vitro grown neural tissues fabricated with vital cells, including exogenous neural cells and their progenitor or stem cells [3–5]. To perform this ambitious task, rapid and effective tissue fabrication techniques are needed to produce functional neural tissues.

In recent years, the inkjet printing technology has been of great interests in tissue engineering, as it is offering a practical and efficient tool to dependably dispense and handle biological and/or material elements in tunable manners to generate cellular and tissue structures or even organ analogs [6,7]. A variety of proteins [8,9], growth factors [10], and even whole viable cells [11,12] have been deposited with the inkjet printing method mainly with the aim of constructing cell patterns. In the field of neuroengineering and neuroscience it may be of particular interest to generate neural cell-based patterns and structures. In previous studies, a two-step procedure was employed, in which the cell-adhesive molecules, such as collagen [8], collagen/poly d-lysine [9], or laminin [13], were printed first, onto substrates with cell-repulsive backgrounds...
(e.g. agarose [8], poly(ethylene) glycol [9]). Subsequently, the neural cells of interest were seeded and formed cellular patterns through preferable attachments to the cell-adhesive molecules. Although these two-step methods have obvious advantages of high throughput, flexibility and cost-effectiveness, they are limited to 2D neuronal patterning due to the use of thin coating of cell-adhesive molecules, and there is difficulty to rebuild 3D complex neural tissue-like constructs.

To overcome this and other limitations, we have successfully expanded the capability of the inkjet printer to eject living cells directly [11,12]. By the direct printing of motoneurons, patterns with specific forms have been generated [12]. Although in the previous study the possibility of inkjet printing of neurons and the viability of printed cells have been verified [12], it was unclear whether the neurons retained cellular properties and function, such as neuronal phenotypes and basic electrophysiology, after being printed. Moreover, the feasibility of simultaneously printing neural cells and biomaterials to build 3D multi-layer neural cell-laden constructs has not been demonstrated.

In previous studies, we showed that cells can survive the inkjet printing process with 90% viability [12]. The hypothesis that is tested in this study is whether the surviving printed neurons develop normal physiological properties, i.e. action potential firing, when compared to non-printed cultures or brain slice experiments. Moreover, this study presents significant improvements in our patterning methods. Furthermore, the hypothesis was tested whether a fast gelling biodegradable material such as fibrin gel can be used to automate the fabrication of 3D structures using the same printing device. In the present study, rat primary embryonic hippocampal and cortical neurons were directly ejected into pre-defined single-layer cellular structures by using a commercial inkjet printer with slight modifications. The immunostaining and the patch-clamp techniques were used to evaluate the phenotypes and electrophysiology of the printed neurons. Furthermore, 3D neural cell-based constructs with specific forms were fabricated by alternately printing fibrin gels and NT2 neurons into specific patterns and 3D structures.

Specifically, we tested the ability of neurons to retain functional fidelity, such as phenotypic properties and electrophysiology, a prerequisite for the development and differentiation of healthy neurons. The thermal ink jetting involves in vaporizing a micrometer-sized layer of liquid in contact with a thin film resistor which may subject the cells to a 5 μs period of heat and stress. In light of the increasing evidence that heat shock has long-term effects on electrophysiological properties of neurons [14,15] and synapses [16], we investigated whether the printing process affects these basic and important properties of neurons by choosing two neuron types derived from different regions of the brain: embryonic hippocampal and cortical neurons.

2. Materials and methods

2.1. Cell preparation and cell print suspension

Primary hippocampal and cortical cells were obtained through enzymatic dispersion of day-18 fetal tissue from pregnant Sprague–Dawley rats as previously described [17,18]. NT2 neuronal precursor cells were obtained from American Type Culture Collection (ATCC 3813555) and cultured in standard conditions (37 °C, 5% CO_2) in Dulbecco’s Modified Eagles Media (DMEM) comprised of 10% fetal bovine serum (FBS), all obtained from Sigma Chemicals (St. Louis, MO). Cell pellets were collected by centrifugation (1000 rpm/5 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) to obtain cell print suspensions of about 2,000,000 cells/ml final concentration. In our previous study we reported an average cell death of 10% due to printing and 15% due to the hypertonic DPBS, thus we expect 75% viability for these experiments. Viability of the printed neurons was estimated by enumerating cells with processes and cells that appeared round and small after 8 days of culture.

2.2. Fabrication of single-layer cellular structures of primary embryonic hippocampal and cortical neurons

Single-layer neural structures with a circle pattern were assembled using the automated and direct inkjet printing process. The HP Desktop 550 printers and HP 51626a ink cartridge were modified to accommodate the delivery of the living cells to fabricate single-layer neuronal patterns as described previously [6]. The printhead of this printer has a face plate with two rows of 25 orifices each ~50 μm in diameter. The orifices in each row are separated by 170 μm and the rows are offset by 85 μm. These printheads are able to position 85 pl drops with a resolution of 85 μm. It takes 200 μs to fire all 50 chambers, resulting in a print speed of up to 250,000 drops per second or in terms of digital fabrication, an hourly throughput of structures with 80 ml volume at 85 μm resolution per printhead [19].

The cartridge was rinsed thoroughly with ethanol and sterile water prior to cell print suspension introduction. A pattern that consisted of rows of circles was designed using Microsoft PowerPoint to program the printer. The bio-paper substrates were prepared from rat-tail Type I collagen gels by using the previously reported protocol [12]. The print suspensions of hippocampal and cortical cells were loaded into the cartridge and the collagen-based bio-papers were placed onto the print stage that is mounted under the printheads. The primary neuronal cells were delivered onto the bio-paper following the pre-designed circle pattern. The printed primary neuron samples were placed into 35 mm dishes and moved to a standard cell culture incubator. After 3–5 h, the neurons began to attach onto the collagen gel-based bio-paper. At that time plating medium consisting of Neurobasal Media (Gibco, Invitrogen, San Diego, CA) with 2% w/v B-27 Supplement 50 × (Gibco), 0.5 mm glutamine (Gibco), and 25 μM glutamate (Gibco), was introduced into the cultures. After 4 days, a maintaining medium was used, which contained the same composition as the plating medium, with the exception of using 25 μM glutamate.

2.3. Immunostaining of printed primary embryonic hippocampal and cortical neurons

 Cultures with the printed hippocampal and cortical neurons were rinsed in 1 × phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 30 min, and rinsed again in PBS. After incubating in the blocking solution (2% bovine serum albumin and 3% goat serum prepared in 1 × PBS) for 30 min at 4-8 °C, printed cortical cells on the collagen gels were exposed to the primary antibody of mouse monoclonal anti-MAP2 (1:50) (Sigma) diluted in PBS overnight at 4-8 °C, while printed hippocampal neurons were exposed to a mixture of two primary antibodies of mouse monoclonal anti-MAP2 (1:50) and rabbit monoclonal anti-neurofilament NF 150 (1:150) (Chemicon, Temecula, CA).
Consequently, fluorescence-labeled secondary antibodies were applied. The stained samples were visualized using a Zeiss LSM-510 confocal microscope.

2.4. Electrophysiology of printed primary embryonic hippocampal and cortical neurons

The electrophysiological characteristics of the printed neurons were evaluated by the patch-clamp technique. Whole-cell patch-clamp recordings were performed in a recording chamber placed on the stage of a Zeiss Axioskope 2 FS Plus upright microscope. The chamber was continuously perfused (2 ml/min) with the extracellular solution (Neurobasal culture medium, pH was adjusted to 7.3 with HEPES, 35 °C). Patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller and filled with intracellular solution (in mM: K-glucuronate 140, EGTA 1, MgCl2 2, Na2ATP 2, Phosphokreatine 5, Phosphokreatine kinase 2.4 mg, HEPES 10; pH = 7.2). The resistance of the electrodes was 6–8 MΩ. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (Axon Inc., Union City, CA) amplifier. Signals were filtered at 2 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface.

Data recording and analysis were performed with pClamp 8 software (Axon). Membrane potential was corrected by the subtraction of 15 mV tip potential, which was calculated using Axon’s pClamp 8 software program. Membrane resistance and capacitance were calculated from a 50 ms voltage steps from −85 to −95 mV without any whole-cell or series resistance compensation. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from −85 mV holding. Whole-cell capacitance and series resistance were compensated and a p/6 protocol was used. The access resistance was less than 22 MΩ. The membrane time constant was determined using 1 ms, −0.8 nA current impulse in current clamp mode. Six traces were averaged and three exponentials were fitted to the averaged trace using the pClamp software. The electrotonic length of neurons was calculated from the decaying phase of the current impulse in current clamp mode. The membrane time constant was measured with 1 s depolarizing current injections from 85 mV holding. Whole-cell capacitance and series resistance were compensated and a p/6 protocol was used. The access resistance was less than 22 MΩ. The membrane time constant was determined using 1 ms, −0.8 nA current impulse in current clamp mode. Six traces were averaged and three exponentials were fitted to the averaged trace using the pClamp software. The electrotonic length of neurons was calculated from the decaying phase of the current impulse.

2.5. Fabrication of 3D neural sheets with fibrin gels

Fibrinogen (Sigma) was dissolved in 1× PBS to make the working solution with final concentration of 10 mg/mL. Bovine thrombin (Sigma) was dissolved in 20μM CaCl2 to get the final concentration of 20 IU/mL and prepared for later inkjet printing.

A thin layer of the fibrinogen was plated onto a clean, sterile coverslip. A print cartridge loaded with the thrombin working solution was mounted into the printer, the thrombin droplets were ejected onto the pre-plated fibrinogen layer for three consecutive times. Fibrin gel formation was observed immediately after thrombin ejection. However, 3–5 min were allowed for fast and partial gelling of the fibrin gels before another cartridge loaded with NT2 neurons was mounted into the printer and the neurons were printed the gelled fibrin. This procedure was then repeated; that is, another thin layer of the fibrinogen was plated onto the previous gelled layer for the subsequent printing of thrombin and NT2 neurons. The whole process of alternate printing of thrombin and NT2 neurons was repeated for 5 cycles resulting in a 3D neural sheet. The printed neural sheet samples were cultured in NT2 differentiation medium, which consisted of DMEM, 10% FBS, and 5% retinoic acid, under standard conditions (37 °C, 5% CO2).

2.6. DAPI staining of the printed 3D neural sheets

After 15 days of culture, the printed 3D neural sheets were fixed in 4% formaldehyde, and DAPI nuclear markers were used to stain the nuclei of NT2 cells within the printed sheet.

2.7. SEMs of the printed 3D neural sheets

The morphology and microstructures of printed NT2 cells and fibrin scaffolds within the 3D neural sheets were examined using a high-resolution SEM. The samples were fixed with 4% formaldehyde in 1× PBS (pH 7.4) for 20–30 min at room temperature. After dehydrating through a graded series of ethanol, the samples were critically dried under vacuum. Then, the samples were sputter-coated with a thin layer of Chromium using a Hummer® 6.2 Sputter Coater (Anatech Ltd) in a 100 mTorr vacuum argon environment for a 3 min period and 10 mA of current. Images were taken using Hitachi S4700N Field Emission Scanning Electron Microscope (Japan) at a 5 kV accelerating voltage.

2.8. Tensile testing of fibrin

The tensile properties of the 3D printed fibrin sheets were determined at room temperature by stretching the sample at a constant deformation rate of 5 mm/min. The un-axial tensile testing was performed by using an electromechanical testing system (MTS Systems Corporation, Eden Prairie, MN) and original data were acquired and analyzed using the software provided with the instrument (TestWorks®). The resulting stress-strain data were used to calculate the linear modulus (LM) and ultimate tensile stress (UTS). The LM was defined as the slope of the linear portion of the stress-strain curve, which occurred in the range of 0–100% of the UTS. The peak stress achieved during mechanical testing was taken as the UTS.

3. Results

3.1. Printed single-layer cellular structures of primary rat embryonic hippocampal and cortical neurons

The single-layer cellular structures with circular patterns of primary rat hippocampal and cortical neurons were fabricated through direct inkjetting of cells. The completed rings of printed rat hippocampal neurons were obvious under a light microscope immediately after printing, as shown in Fig. 1A. The neural cell rings showed strong compliances to the pre-designed circular patterns. After 1 day of culture, the printed neurons began to differentiate and develop their processes. Fig. 1B shows a typical printed culture after 8 days. One can clearly distinguish between differentiating neurons and dead or round cells. Enumerating the dead vs. live cells, a viability of 74.2±6.3% is obtained. Fig. 1C and D shows the polarized morphologies of the printed hippocampal neurons after 13 days of culture and cortical neurons after 9 days of culture, respectively. These cells exhibited normal neuronal appearance and a good growth of neurites on the collagen-based bio-papers. Table 1 summarizes all the printing parameters, the expected and obtained results. It is noted that the printing is very predictable both in terms of deposited cells as well as estimated live cells.

3.2. Immunostaining of printed rat primary embryonic hippocampal and cortical neurons

After incubation with the neuronal-specific markers, anti-MAP-2 and/or anti-neurofilament (NF150) antibodies, the processes and cell bodies of printed brain neurons
Fig. 1. Single-layer cellular structures of rat primary embryonic hippocampal and cortical neurons fabricated by the direct printing process. Light microscopy images of printed embryonic rat hippocampal neuronal ring immediately after printing (A) and on day 8 (B) used to enumerate the number of viable cells. Morphologies of printed hippocampal neurons (C) after 13 days of culture and printed cortical neurons (D) after 9 days of culture, both recorded by phase contrast imaging.

Table 1
Cell density and viability of the hippocampal cells within the printed ring patterns

<table>
<thead>
<tr>
<th>Specification of the printer</th>
<th>Firing frequency</th>
<th>5 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution</td>
<td>85 μm (300 dpi)</td>
<td>[29]</td>
</tr>
<tr>
<td>Drop volume</td>
<td>85 pl [30]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Printing parameter</th>
<th>Concentration of cell print suspension</th>
<th>2,000,000 cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of printed dots</td>
<td>9996</td>
<td></td>
</tr>
<tr>
<td>Volume of ejected drops</td>
<td>0.85 μl</td>
<td></td>
</tr>
<tr>
<td>Area of the “O” ring pattern</td>
<td>7.61 mm²</td>
<td></td>
</tr>
<tr>
<td>Time to print the pattern</td>
<td>2 s</td>
<td></td>
</tr>
<tr>
<td>Average number of the cells per ejected dropa</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Designed (or expected) value</th>
<th>Number of printed cellsb</th>
<th>1700</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell density within the patternc</td>
<td>223 cells/mm²</td>
<td></td>
</tr>
<tr>
<td>Viability of the neurons within the patternd</td>
<td>&gt;76.2%e</td>
<td></td>
</tr>
<tr>
<td>Living cell density within the patternf</td>
<td>&gt;170 cells/mm²</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimental values</th>
<th>Total cell density within the pattern at day 8 (n = 4)</th>
<th>250 ± 29 cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Living cell density within the pattern at day 8 (n = 4)f</td>
<td>184 ± 10 cells/mm²</td>
</tr>
<tr>
<td></td>
<td>Viability of the neurons within the pattern (n = 4)</td>
<td>74.2 ± 6.3%</td>
</tr>
</tbody>
</table>

aNumber of cells in single ejected drop = droplet volume × concentration of cell print suspension.
bNumber of printed cells = Number of printed dots × Number of cells per drop.
cTotal cell density within the pattern = Number of printed cells/Area of the pattern.
dFrom Ref. [12].
eLiving cell density = total cell density × viability.
fOnly the neurons which obviously exhibited their processes and differentiated were counted as the living cells after 8 days of culture because after this relatively long-term period of culture, neurons derived from embryonic tissue go through their normal developmental stages and develop their processes.
exhibited strong immunoreactivity and emitted specific fluorescence under excitation laser sources. Fig. 2 shows confocal microscope images of printed cortical and hippocampal neurons after immunostaining for MAP-2 and double immunostaining for MAP-2 and neurofilament (NF150), respectively. The dendrites and cell bodies of printed cortical cells were stained positive and exhibited green fluorescence (Fig. 2A). In addition to their dendrites and cell bodies staining positive (green), the axons of printed hippocampal cells were stained positive (red) (Fig. 2B).

### 3.3. Electrophysiology of printed primary embryonic hippocampal and cortical neurons

Electrophysiological recordings were performed on the printed primary hippocampal and cortical neurons after 2 weeks in culture [5]. To examine the development of voltage-gated ion channels within the printed primary neurons, the whole-cell voltage clamp mode was used to record K⁺ and Na⁺ currents. As shown in Fig. 3A and B, the voltage-gated outward K⁺ currents and inward Na⁺ currents were recorded from the printed hippocampal and cortical neurons after 15 days in culture, and these currents could be consistently evoked by 10 mV steps from a hold potential of −85 mV. These recordings suggest that the printed cortical cells had developed into mature neurons with voltage-gated potassium and sodium channels on the membranes.

Moreover, to examine the excitability of these printed neural cells, the current clamp model was performed and the sustained repetitive firings of action potentials were observed from the two printed cells, which indicated the printed cortical and hippocampal cells were excitable and capable of firing action potentials. These patch-clamp recordings are shown in Fig. 3C and D for cortical and hippocampal cells, respectively. Additionally, the passive or active membrane properties of the printed hippocampal cells and controls (manually plated) were compared as shown in Table 2. All of the measured membrane properties did not show any statistically significant differences between printed hippocampal cells and the controls (Two-sample student’s t-test, $P > 0.05$). In addition, the data are in good agreement with the published results for the emergence of the normal electrophysiology of the embryonic hippocampal neurons [9,20–22].

### 3.4. Fabrication of 3D neural sheets

The 3D neural constructs were fabricated by direct printing of NT2 neurons and fibrin gels using a layer-by-layer approach. The thickness of each layer as calculated by dividing the total thickness of the gel by the number of printed layers ranges from 50 to 70 µm. The printed gels had an LM of $2.92 \pm 0.82$ MPa and ultimate tensile strength of $1.7 \pm 0.5$ MPa ($n = 3$), which falls within the range of properties reported for unligated fibrin clots [23]. The constructs exhibited a 3D sheet form with the gross dimension of $25 \text{mm} \times 5 \text{mm} \times 1 \text{mm}$ shown in Fig. 4A. Many nuclei stained blue following the DAPI staining. Fig. 4B shows a representative sample of a large number of NT2 cells entrapped and distributed evenly within the printed neural sheets. Under light microscopy, most NT2 cells were observed to spread over the fibrin and some exhibited the outgrowth of neuritis after 12 days of culture, as shown in Fig. 4C.

The interactions between the spread NT2 cells and the fibrin fibers and the microstructures of the scaffolds within the printed 3D neural sheets were examined by high-resolution SEM. As shown in Fig. 4D, the anchors of the filopodia of those cells to the fibrin fibers were observed, indicative of the attachments of the NT2 cells to the printed fibrin gels. Fig. 4E shows the porosity of the printed fibrin gels and the morphology of fibrin fibers. The average diameter of the fibrin fibers was measured to be $0.1096 \pm 0.0200 \text{µm}$ ($n = 5$). The formed fibrin scaffolds
exhibited a loose and porous microstructure, which may provide an efficient system to supply nutrients and oxygen to the entrapped NT2 cells.

4. Discussion

4.1. Maintenances of phenotypic and electrophysiological characteristics of printed primary neurons

A prerequisite for building functional neural structures and tissues by the inkjet method is that the printed neurons should display normal, healthy physiological properties and functions. The thermal inkjet printing is often seen as a process which can involve high temperatures (up to 300 °C) and sheering stress (10 m s\(^{-1}\)) \[11,12\]. Although we previously demonstrated the possibility of direct printing of living cells and more than 90% mammalian cells were not lysed during thermal printing \[12\], the influences of the printing process on basic cellular properties and functions of printed cells has not been studied. One main concern is whether after being printed through firing nozzles the primary neurons could lose neuronal phenotypes and change to other cell types (metaplasia), like glial cells or even worse to tumor cells, instead of going through their proper development stages. Another concern is whether printed neurons could retain normal electrophysiological properties and become functional.

In this study two well-established neuronal markers, MAP2 and neurofilament were used for the identification of the cell bodies and dendrites vs. axons of the printed hippocampal and cortical neurons, respectively. Immunostaining for dendritic marker MAP-2 and/or axonal filament marker NF150 (Fig. 2) showed the printed hippocampal and cortical neurons had regrown both axonal and dendritic processes. Axon and dendrite regeneration is important to the proper development of printed neural cells. The results of immunostaining strongly suggested the development of neuronal polarity and the maintenance of neuronal phenotypes of these neurons after being printed through thermal inkjet nozzles.

Fig. 3. Electrophysiological characterizations of rat embryonic hippocampal and cortical neurons after being printed through firing nozzles. Representative recordings of sodium and potassium currents obtained from a day-16 hippocampal neuron (A) and a day-15 cortical neuron (B). Maximum action potential firing rates of a day-16 hippocampal neuron (C) and a day-15 cortical neuron (D).
Table 2
Passive and active membrane properties of embryonic hippocampal neurons after being printed through firing nozzles

<table>
<thead>
<tr>
<th>Membrane properties of hippocampal cells at day 15</th>
<th>Printed</th>
<th>STD</th>
<th>Control</th>
<th>STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_m$ (mV)</td>
<td>-35.7</td>
<td>3.6</td>
<td>-35.78</td>
<td>5.76</td>
</tr>
<tr>
<td>$R_S$ (MΩ)</td>
<td>382</td>
<td>185</td>
<td>220</td>
<td>92</td>
</tr>
<tr>
<td>$C_M$ (pF)</td>
<td>45.8</td>
<td>16.3</td>
<td>32.4</td>
<td>16.7</td>
</tr>
<tr>
<td>$L$ $\tau_0$ (ms)</td>
<td>1.80</td>
<td>0.31</td>
<td>1.86</td>
<td>0.45</td>
</tr>
<tr>
<td>$\tau_0$ (ms)</td>
<td>22.74</td>
<td>8.73</td>
<td>11.11</td>
<td>5.26</td>
</tr>
<tr>
<td>$T_{VC1}$ (ms)</td>
<td>0.52</td>
<td>0.25</td>
<td>1.01</td>
<td>0.4</td>
</tr>
<tr>
<td>$Na$ current (pA)</td>
<td>-2390</td>
<td>957</td>
<td>-3455</td>
<td>897</td>
</tr>
<tr>
<td>$K$ current (pA)</td>
<td>2569</td>
<td>1018</td>
<td>2439</td>
<td>709</td>
</tr>
<tr>
<td>$V_{th}$ (mv)</td>
<td>-46.9</td>
<td>1.5</td>
<td>-47.7</td>
<td>4.6</td>
</tr>
<tr>
<td>$I_{th}$ (pA)</td>
<td>61.3</td>
<td>35.6</td>
<td>108</td>
<td>22.8</td>
</tr>
<tr>
<td>Firing type</td>
<td>1.22</td>
<td>1.86</td>
<td>1.86</td>
<td>1.46</td>
</tr>
<tr>
<td>Max firing (Hz)</td>
<td>2.44</td>
<td>2.24</td>
<td>3.71</td>
<td>5.62</td>
</tr>
<tr>
<td>AP Ampl. (mV)</td>
<td>18.8</td>
<td>8.4</td>
<td>13.1</td>
<td>12.9</td>
</tr>
<tr>
<td>AP Dur. (ms)</td>
<td>2.95</td>
<td>0.77</td>
<td>5.08</td>
<td>1.95</td>
</tr>
<tr>
<td>AHP Ampl. (mV)</td>
<td>-4.72</td>
<td>0.57</td>
<td>-4.08</td>
<td>0.97</td>
</tr>
<tr>
<td>AHP Dur. (ms)</td>
<td>74.5</td>
<td>31.7</td>
<td>59.4</td>
<td>19.51</td>
</tr>
<tr>
<td>$N$</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Electrophysiological parameters were measured using conventional voltage clamp and current clamp protocols during whole-cell patch-clamp recordings from printed hippocampal neurons. $V_m$: resting membrane potential; $R_S$: input resistance; $C_M$: membrane capacitance; $L$: electrotonic length; $\tau_0$: zero-order membrane time constant; $T_{VC1}$: longest membrane time constant in voltage clamp mode; $I_{th}$: action potential threshold voltage; $V_{th}$: action potential threshold current; Firing type: empirical classification neurons (0 from 4: category 0, no action potential; category 1, 1 or 2 action potentials; category 3, repetitive firing but duration of the firing is less than 1 s; category 4, continuous repetitive firing) based on their repetitive firing properties.

As described above on the results of the patch-clamp experiments, both printed hippocampal and cortical cells exhibited normal recordings of voltage-gated outward $K^+$ currents and inward $Na^+$ currents, suggesting these cells expressing voltage-gated potassium and sodium channels on the membranes. Moreover, the recordings of sustained repetitive firings of action potentials indicated that the printed hippocampal and cortical cells were excitable and capable of firing action potentials. Additionally, the printed hippocampal cells exhibited normal passive membrane properties, as seen by the fact that there were no significant differences between the measured parameters in the printed and the control group. The electrophysiological results proved that the primary neurons maintained normal electrophysiological properties after being printed.

Thermal printing may not only result in the survivals of the printed cells as we described previously [11,12], but also give rise to the maintenance of phenotypic and electrophysiological characteristics of the printed primary neurons as we demonstrated in this study. All the data presented here, without surprise, confirm our previous analysis that the droplet ejection process does not cause significant cell damage [8,11,12]. In fact, the heat is only applied in the droplet formation stage which lasts for less than 3 μs, a timeframe during which the heat transfer into the liquid bulk of the droplet causes only a marginal rise in temperature (4–10 °C). In addition, this timeframe is 5 orders of magnitude faster than typical response of neurons which are considered fast responders to environmental factors. Thus we suggest that the cells are unable to respond to such fast transients as experienced in the inkjet nozzles.

The shear experienced by the cells as they are ejected though the nozzles was another concern since it has been shown previously that even small shear stresses can cause damage in differentiated neurons [24]. Here we used trypsinized cells that had lost their internal architecture and cytoskeletons at the time of printing. It may be possible that these cells without their internal architecture may be less vulnerable to stress. In fact, stress stimuli can elicit two distinct reactive cellular responses: the heat shock response and the activation of cell death pathways [14,25]. Our data on cell viability and neural cell differentiation are supporting the fact that printing elicits little if any, apoptosis. We do not have direct evidences on the possible expression of heat shock proteins in printed neurons. However, there is increasing evidence that heat shock has long-term effects on electrophysiological properties of neurons [15,16] and synapses [16]. Compared with their respective controls (normal neurons), some heat shocked neurons exhibited different intrinsic excitability properties, such as membrane resistance, resting membrane potential, firing frequency [16], amplitude [16,26], and duration [15] of action potentials. In the electrophysiological study presented here, there were no significant differences between printed cells and their controls (normal) on those neural basic properties. Thus we conclude that the printed neurons exhibited normal electrophysiology and the heat shock responses may not have occurred. The absence of heat shock response and the absence of apoptosis after the printing process further suggest that cells were unable to mount such responses due to the micro-second timeframe of the firing process.

4.2. Printable fibrin hydrogel

In this study, fibrin was used as a printable hydrogel to build 3D neural constructs. Fibrin is a biopolymeric gel that plays an important role in natural wound healing [27]. Recently, fibrin has been applied into tissue engineering applications as an important scaffold material for engineered cardiovascular and neural tissues [27]. The fibrin gel is formed by the enzymatic polymerization of fibrinogen. Initially, the protease thrombin cleaves dimeric fibrinogen molecules at two symmetric sites. Once the fibrinogen is cleaved, a self-assembly step occurs in which the fibrinogen monomers come together and form a non-covalently cross-linked polymer gel via the proteolytic exposure of binding sites. Since the whole process of gelling is very fast, it may present an alternative material for inkjet printing. Printing of thrombin into fibrinogen will cause geometry-specific
cross-linking, thus enabling the rapid construction of 3D fibrin scaffolds with specific structures and forms.

Another important reason to use fibrin gel to build 3D neural constructs lies in the affinity of neurons for the gel. Neurons are anchorage-dependent and their functionality depends on their attachment onto scaffolds. Without attachment to scaffolds, the neurons cannot develop and differentiate. Cells receive important signals from ECM which play a critical role in cell development and survival. Due to the anchorage dependence of neurons for growth and survival, any disruption of cell attachment can lead to the interruption of these signals causing stress to the cells and eventually leading to apoptosis [18]. Some other anchorage-dependent cells have shown the ability to be attached onto the fibrin gel. For example, it has been demonstrated that the fibrin can be used as a natural ingrowth matrix for endothelial cells [28]. Isolated human mesenchymal stem cells were also demonstrated to attach on the fibrin gel when appropriate concentrations of fibrinogen and thrombin were used. In this study the attachment of NT2 neurons to the fibrin gel was demonstrated (Fig. 4D).

Moreover, the fibrin gel has other obvious advantages. For examples, fibrinogen and thrombin are blood-borne proteins and can easily be purified from blood, offering the opportunity of using an autologous source for the scaffold and eliminating immunological concerns of human or cross-species donor incompatibility [28]. Another special feature of fibrin, both formed naturally or therapeutically, is that it is degraded and remodeled by cell-associated

Fig. 4. 3D neural sheets were fabricated by alternately printing fibrin gels and NT2 cells. (A) Photography of a printed neural 3D sheet in the culture medium at day 1. The gross dimension of the 3D neural sheet was 25 mm × 5 mm × 1 mm. (B) DAPI staining of NT2 cells within the printed sheet after 15 days of culture. The nuclei of NT2 cells were stained to be purple under a fluorescence microscopy. (C) Microscopy images of NT2 cells within the printed neural 3D sheet (phase contrast). NT2 cells within the fibrin gel developed their processes out after 12 days of culture. (D) The attachment of an individual NT2 neuron onto the fibrin fibers was seen in the neural sheet. (E) SEMs of the fibrin scaffolds of the neural sheets exhibited the morphologies of fibrin fibers and porosities of the fibrin gel. Original magnifications are as follows: 3500 × (D) and 5000 × (E).
enzymatic activity during cell migration and wound healing. The degradation of fibrin gel is easily controlled by aprotinin, a proteinase inhibitor to slow down or stop fibrinolysis. In addition, in vitro studies recently reported that fibrin gels might promote cell migration, proliferation, and matrix synthesis through the release of platelet-derived growth factors and the transforming growth factor beta [27]. With those characteristics of the fibrin gel, the printed fibrin-based neural constructs could provide suitable cell or tissues sources with in-vivo affinity for clinical treatments of serious neural injuries and degenerative diseases, such as spinal cord injury and Parkinson’s disease.

5. Conclusions

To the best of our knowledge, this study was the first to evaluate cellular properties and functional fidelity of neurons, including neuronal phenotypes and electrophysiology, after being ejected through the nozzles of a thermal inkjet printer. The results presented here showed that controlled patterns and structures of primary embryonic hippocampal and cortical neurons can be fabricated by the novel inkjet printing method. Furthermore, the maintenance of neuronal phenotypes and basic electrophysiological functions of the hippocampal and cortical neurons was verified. Additionally, the biodegradable fibrins as fast-gelling gels were induced into the inkjet printing process and 3D neural sheets were generated by alternate printing of fibrin gels and NT2 cells. This layer-by-layer inkjet printing method offers the capability of the modified inkjet printers to fabricate 3D functional neural tissues.

References