Three-dimensional bioprinting of rat embryonic neural cells

Wonhye Lee[a,b], Jason Pinckney[a], Vivian Lee[b], Jong-Hwan Lee[a], Krisztina Fischer[a], Samuel Polio[a], Je-Kyun Park[b] and Seung-Schik Yoo[a,b]

We present a direct cell printing technique to pattern neural cells in a three-dimensional (3D) multilayered collagen gel. A layer of collagen precursor was printed to provide a scaffold for the cells, and the rat embryonic neurons and astrocytes were subsequently printed on the layer. A solution of sodium bicarbonate was applied to the cell containing collagen layer as nebulized aerosols, which allowed the gelation of the collagen. This process was repeated layer-by-layer to construct the 3D cell–hydrogel composites. Upon characterizing the relationship between printing resolutions and the growth of printed neural cells, single/multiple layers of neural cell–hydrogel composites were constructed and cultured. The on-demand capability to print neural cells in a multilayered hydrogel scaffold offers flexibility in generating artificial 3D neural tissue composites. *NeuroReport* **20:798–803 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

NeuroReport 2009, **20**:798–803

Keywords: astrocytes, cell printing, collagen hydrogel, freeform fabrication, neurons, tissue engineering

[a]Department of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA and [b]Department of Bio and Brain Engineering, College of Life Science and Bioengineering, KAIST, Daejeon, Republic of Korea

Correspondence to Dr Seung-Schik Yoo, PhD, MBA, Department of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA
Tel: +1 617 525 3308; e-mail: yoo@bwh.harvard.edu

Received 8 January 2009 **accepted** 11 March 2009

Introduction

Patterning of neural cells has been used to construct artificial neural tissues for the investigation of pathogenesis/repair of neurological diseases or to study the properties of neural networks [1]. With advances in cell patterning techniques such as softlithography and microfluidics, neural cells can be plated in a microsized chamber [2] or positioned to form a specific shape by microstamping neuron-repulsive/adhesive materials [3]. However, these techniques lack flexibility in patterning cells in three-dimensions and require a fixed-form mold to position cells and other cell constructs. The three-dimensional (3D) cellular structure is crucial for the recreation of the cell-to-cell, or cell-to-extracellular matrix interactions that are necessary for normal tissue function and development. Stratified layers of brain cortices or ascending/descending neural pathways arranged in the spinal cord are examples of such structures.

Freeform fabrication, also known as rapid prototyping, has recently been applied to assemble user-defined 3D structures that are constructed layer-by-layer [4]. Computer-aided-design software is typically used for transforming the cross-sectional plane of a 3D object into individual 2-dimensional layers. Recently, freeform fabrication technology has emerged as a new means to create an engineered tissue whereby cells and other tissue constructs are dispensed and patterned into the desired spatial locations without the use of a lithographic mold [5]. Neural cells have been patterned onto a planar hydrogel scaffold based on an inkjet printing method [5,6]; however, multilayered, on-demand construction of 3D structures with neural cells, in vitro, has not been reported to our knowledge.

One of the hurdles preventing multilayered construction of a hydrogel–cell composite is that hydrogel precursors (before gelation), printed or injected, are prone to being washed away or distorted during the application of the crosslinking material [5]. We developed a novel method to print collagen hydrogel precursor (in liquid form) and sequentially crosslink the hydrogel while conserving the printed morphology through the application of a nebulized crosslinker, sodium bicarbonate. Neural cells were also printed and embedded in layers of collagen before gelation.

Materials and methods

Overview of the robotic 3-dimensional bioprinter and preparation of astrocytes and neurons

The 3D bioprinter consisted of a 4-channel dispenser array, syringe reservoirs/tubing, a planar (2-dimensional) robotic stage, which mounted the dispenser array, and a vertical robotic stage on which the substrates were located. The detailed specifications and operating principle of the bioprinter are described elsewhere [7].

Astrocytes and neurons from embryonic rat (day 18; BrainBits LLC, Springfield, Illinois, USA) were prepared according to the vendor's protocol. The neurons were suspended in the media and loaded into the syringe after dilution to a concentration of 3×10^6 cells/ml. The

0959-4965 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/WNR.0b013e32832b8be4

astrocytes were subcultured (passage 3) and loaded into another syringe at a concentration of 1×10^6 cells/ml. A concentration of cells greater than 5×10^6 cells/ml was avoided owing to the cell aggregation and the potential clogging of the dispensing nozzle. The syringes containing the neural cell suspensions were gently vibrated to prevent cell aggregation. The viability of the printed neural cells was first examined. Neural cells were printed directly onto a 96 multiwell plate coated with poly-D-lysine (Sigma, St. Louis, Missouri, USA). As a control, the cells were manually plated on the surface of the wells. A viability/cytotoxicity assay was performed for the neural cells a day later using a live/dead assay kit (Molecular Probes, Massachusetts, USA).

Preparation of collagen hydrogel precursor

Rat tail origin type I collagen (BD Biosciences, Massachusetts, USA) was used as a hydrogel precursor for the scaffold. First, the collagen precursor was diluted to 1.12 mg/ml with 0.02 N acetic acid solution and 1X phosphate-buffered saline (Gibco, New York, USA) (volume ratio of 1 : 1 : 2; final pH=4.5) and kept on ice. According to a previous study by Krewson *et al.* [8], which examined the different collagen concentrations to culture neurons, a degree of optimization of collagen scaffold concentration is needed to ensure the proper neurite outgrowth while preserving the mechanical integrity of the scaffold itself. The dilution factor was determined from the collagen density that showed the most significant neurite outgrowth among three different densities of collagen (2.23, 1.49 and, 1.12 mg/ml). A collagen concentration lower than 1.12 mg/ml affected the integrity of the collagen gel in the media as the collagen hydrogel partially dissolved into the media.

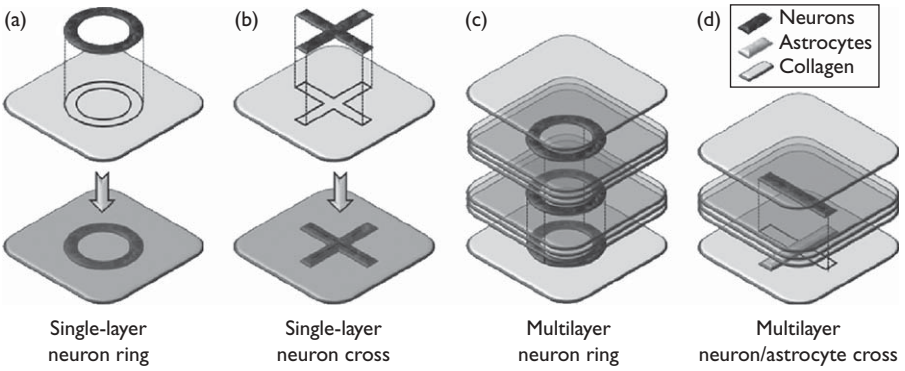
Method of constructing single/multilayered cell–hydrogel composites

To construct multilayer cell–hydrogel composites, a printed hydrogel precursor layer was crosslinked to form a hydrogel before printing any subsequent layers. Sodium bicarbonate solution (0.8 M in distilled water) was used to neutralize (and thus, crosslink) the acidic collagen precursor solution. First, the surface of the tissue culture dish was coated with sodium bicarbonate solution that was nebulized by an ultrasonic transducer (SU-1051W, Sunpentown, California, USA). During this process, the generation of ultrafine mists with droplets less than 2 μm in diameter (when landed on the culture dish surface, as measured by microscope) was crucial to crosslink the dispensed collagen precursors (in the order of 200–300 μm in diameter) without distorting the printing morphology because of the surface tension of dispensed droplets [7]. The cell suspension was then printed on the partially-crosslinked hydrogel layer to lodge the cells inside the collagen. A coating of nebulized sodium bicarbonate solution was then applied to the surface to crosslink the remainder of the collagen layer. The process was repeated to construct multiple layers of collagen and cells. Each collagen layer was printed to occupy a $10 \times 10\,mm^2$ area using the interdispensing distance (i.e. printing resolution) of 600 μm.

Testing of neural cell printing resolutions

Before the multilayered neural cell–hydrogel printing, the relationship between printing resolution and the growth tendency of cells was investigated. Six different printing resolutions (150–400 μm in 50 μm step) were examined for printing neurons in a single layer of collagen (measuring $5 \times 5\,mm^2$; $n=3$), whereas three different printing resolutions (200, 400, and 600 μm) were examined for astrocytes. After printing, the cells were monitored using bright field microscopy (for astrocytes) or green fluorescent live staining (Calcein AM; for visualization of neurites through the semitransparent collagen scaffold). Based on the examination of growth pattern (Fig. 2), a resolution of 150 μm for neurons and 300 μm for astrocytes were selected for subsequent printing experiments.

Fig. 1



Schematics of single-layer and multilayer patterning of neuronal cells (a) a 'ring' pattern of neurons with a 3 mm diameter and (b) a 'cross' pattern of neurons with 6 mm long in a single collagen layer. The multilayer ring patterns of neurons (c) and a multilayered 'cross' pattern consisted of astrocytes and neurons (d) are also illustrated.

Printing and culture of neural cells in single-layered and multilayered hydrogel scaffolds

Neurons were printed and cultured in a 'ring' pattern (3 mm diameter; Fig. 1a) and a 'cross' pattern (two 6 mm-long orthogonal lines; Fig. 1b). To generate multilayered cell–hydrogel composites, a total of eight layers of collagen were printed (Fig. 1c). Rings of neurons were separated by the two layers of collagen, which were sandwiched between printed rings of neurons. We also printed a multilayered 'cross' pattern consisting of astrocytes and neurons (Fig. 1d). To test the feasibility of printing two types of cells into the same area for coculture, both astrocytes and neurons were printed as a single layer in the middle of the collagen scaffold ($3 \times 3\,mm^2$). Neurons were printed at slightly lower resolution (200 μm) to account for the added astrocytes.

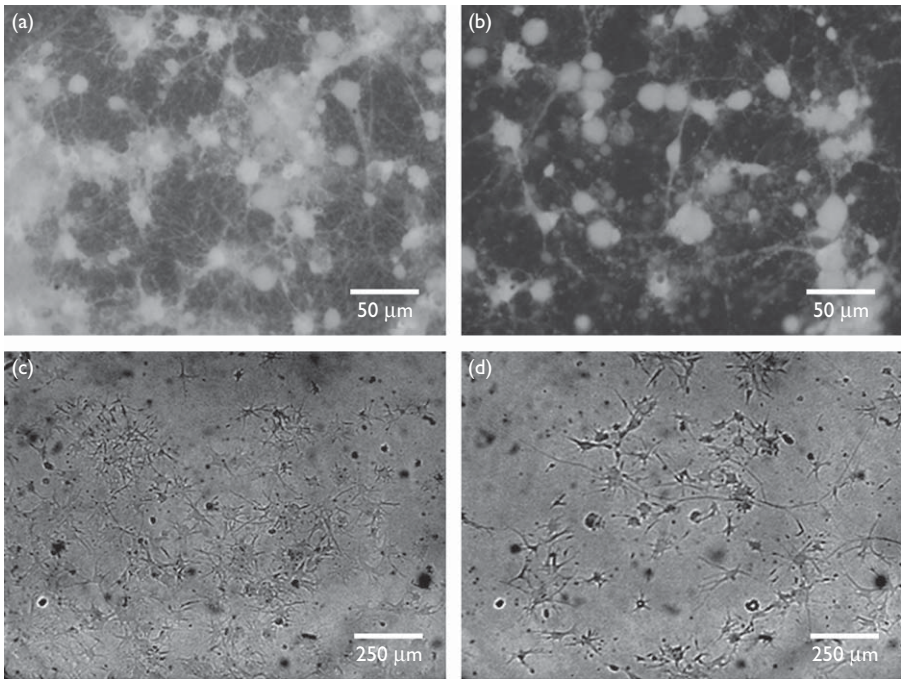
After printing, the neural cell–collagen composites were cultured at 37°C and 5% CO_2 in Neurobasal media with 2% B27 supplement, 0.5 mM glutamine, and 25 μM glutamate. Half of the media was replenished with fresh media (without glutamate) every 3 or 4 days, and the cells were cultured for a maximum of 15 days. The printed cell–collagen composites were immunostained using microtubule-associated proteins 2 (dilution factor 1 : 250; Santa Cruz Biotechnology, Inc., California, USA) for labeling neurons and Glial fibrillary acidic protein (1 : 200; Santa Cruz Biotechnology, Inc.) for labeling astrocytes according to the vendor-suggested protocol (*http://www.cellsignal.com/support/protocols/IF.html*). Subsequently, Texas Red fluorescence-labeled secondary antibody (1 : 100; donkey antirabbit, Jackson Laboratories, Inc., Pennsylvania, USA) was applied for labeling the neurons. Fluorescein isothiocyanate fluorescence-labeled secondary antibody (1 : 100; goat anti-mouse, Jackson Laboratories) was used for the astrocytes. To increase the penetration of antibodies into the scaffold, the sample was placed on a rocker (frequency 30 rpm) during all procedures without using any cover slide. To visualize the neurite outgrowth in a thick (in the order for several hundred micrometers) and semitransparent hydrogel, we adopted the visualization method proposed by O'Connor *et al.* [9] and Othon *et al.* [10] whereby the 'stacks' of multisliced confocal images (Obtained from LSM 510 confocal with two-photon, Carl Zeiss, Germany) were digitally projected along the vertical direction (so called 'Z-stacking' technique) to capture the 3D representation of the cell morphology..

Results

Droplet size and viability assay of printed neural cells

When measured through high-speed camera (Pixelink, Canada), the droplet volume of dispensed cell suspension and collagen precursor was approximately 11 and 8 nl, respectively. The number of cells contained in each droplet was 217.8 ± 21.6 cells for neurons ($n=12$) and 49.8 ± 4 cells for astrocytes ($n=4$). The viability of neurons (control) was $75.2 \pm 2.3\%$ ($n=32$) while printed

Fig. 2



Fluorescent images of printed neurons in single layer of collagen scaffold at printing resolutions of (a) 150 μm and (b) 250 μm were taken after day 15. Bright field images of printed astrocytes in single layer of collagen scaffold with printing resolutions of (c) 400 μm and (d) 600 μm after day 3.

neurons showed a viability of 78.6 ± 0.6% (n=34). The viability of astrocytes (control) was 78.7 ± 5.3% (n=12) while printed astrocytes showed a viability of 78.1 ± 10.0% (n=12). There was no significant difference in the viability of printed neural cells compared with the control group ($P > 0.05$; t-test, two-tailed), suggesting that the cell printing did not affect cell viability.

Investigation of printing spatial resolutions

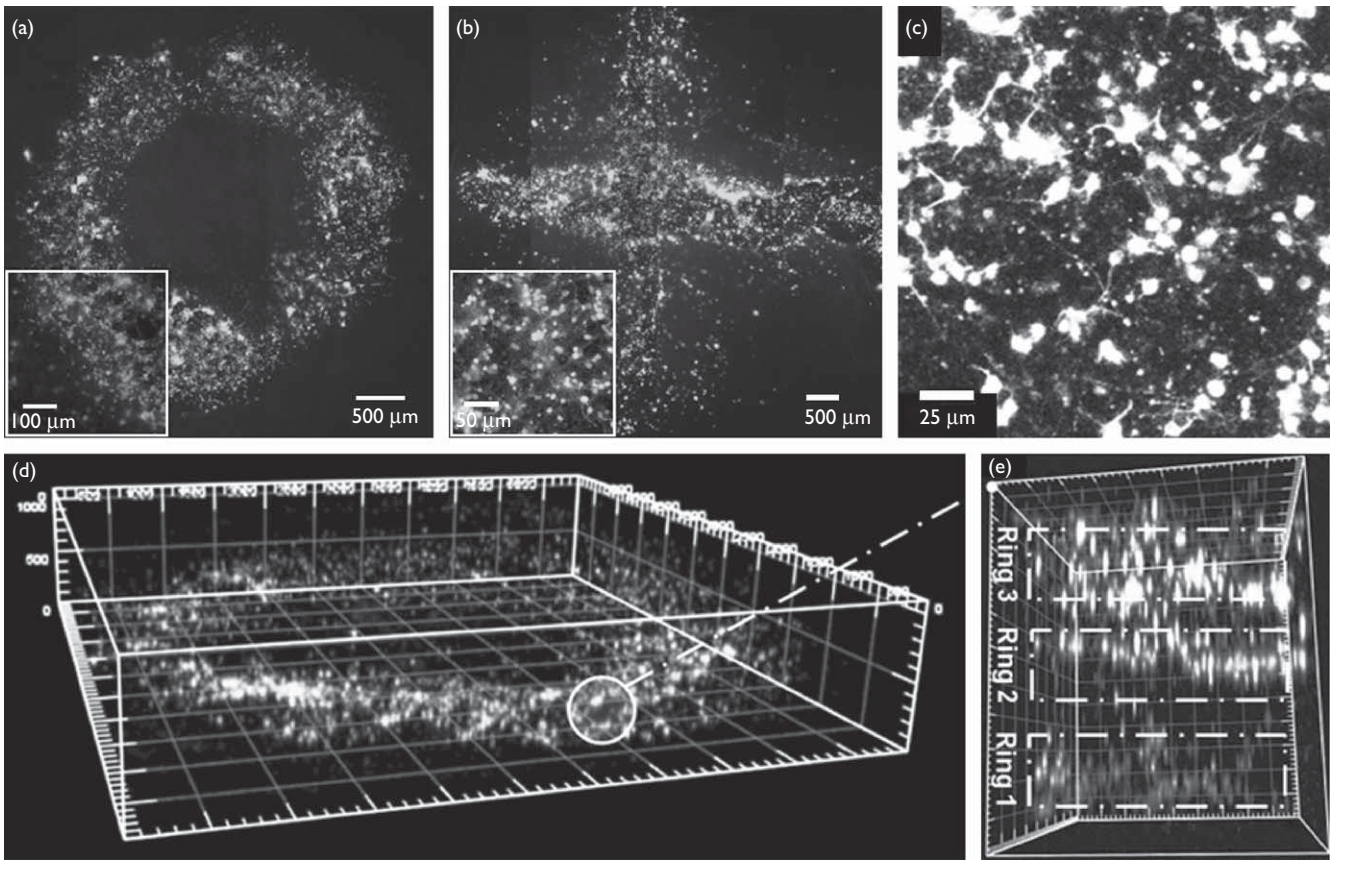
Day 15 culture images of Fig. 2a and b showed a difference in density of cultured neurons at printing resolutions of 150 and 250 μm interdispensing distance. The neurons printed at 250 μm resolution (Fig. 2b) were more sparsely distributed compared with the neurons printed at 150 μm resolution (Fig. 2a), which showed the elevated cell density and neural connectivity through neurite outgrowth. The neurons printed at a low printing resolution did not display visible neurite outgrowth within 10 days. The astrocytes printed at a resolution of 600 μm showed a slow growth rate (Fig. 2d) compared with the ones printed at a resolution of 200 μm, which reached excessive confluency. However, printing resolution of 400 μm lead to a sufficient growth rate and morphologies of astrocytes (Fig. 2c).

Culture of printed neural cells in single-layered and multilayered collagen scaffold

Mosaic fluorescent images of the printed neural cells are shown in Fig. 3. The ring and cross pattern of live neurons in a collagen layer are shown in Fig. 3a and b, respectively. The multilayer pattern of three neuron rings was shown in a 3D-rendered microtubule-associated proteins 2 immunostaining image (Fig. 3d). As evident from the reconstructed side view (inset Fig. 3e), three distinct layers of rings of neurons were distinguished. Patterned neurons showed neurite outgrowth and neural connectivity in three dimensions, based on the projected multistack confocal image (Fig. 3c).

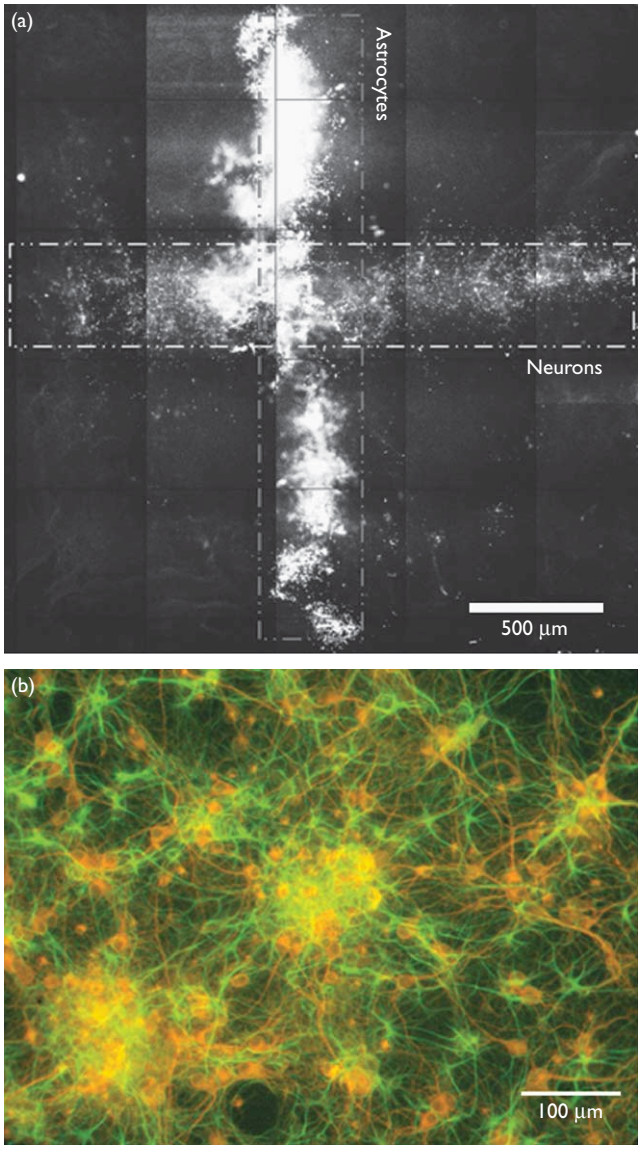
The immunostaining results obtained from the neurons and astrocytes that were printed on a single-layer collagen scaffold were shown in Fig. 4. As anticipated, the star-like

Fig. 3



Fluorescent live-staining images of cultured neurons in a single-layered collagen scaffold on day15 after printing. (a) A printed 'ring' pattern of neurons with 3 mm diameter in a single layer of collagen scaffold (Inset: a part of the printed 'ring'). (b) A printed 'cross' pattern of neurons 6 mm long in a single layer of collagen (Inset: a part of the printed 'cross'). (c) Vertically-projected image of printed cell block in collagen through 3D volume. (d) Multilayer patterning of three neuron rings within eight layers of collagen. A magnified side view (e) shows distinct layers of printed neurons.

Fig. 4



Immunostaining images of printed neurons (microtubule-associated proteins 2-labeled, in orange) and astrocytes (Glial fibrillary acidic protein-labeled, in green) in a single-layer collagen scaffold after day 12 of culture. (a) 3D multilayered patterning of the cells in six layers of collagen (4′-6-Diamidino-2-phenylindole staining; imaged on day 7). The vertical line composed of astrocytes located in first collagen layer and horizontal line composed of neurons embedded in the fifth collagen layer (counted from bottom). (b) Cocultured neurons and astrocytes in a single-layer collagen scaffold.

morphology of astrocytes, which is typically observed on planar substrates, was slightly distorted in the volumetric collagen gel [11]. Figure 4a shows a multilayered pattern of neurons and astrocytes stained with 4′-6-Diamidino-2-phenylindole staining to visualize the macroscopic location of the printed cells through a thick hydrogel scaffold. The clusters of both neurons and astrocytes were visible in the middle as well as in the lower left corner of Fig. 4b.

Discussion

Tissue engineering aims to replace damaged or defective tissues with artificially-engineered tissue products made of naturally-driven or synthetic biomaterials with strategic placement of appropriate cells exhibiting normal functional potency. By constructing an artificial tissue from an assembly of cells and necessary biomaterials, tissue-engineering techniques can offer 3D cellular environments that mimic the natural physiological/geometrical conditions of healthy tissue. Other than applications in medical therapy, there have also been increasing efforts to grow neural cells in vitro for drug-testing platforms [12], neuroscientific research on neural networks [1,13], the development of retinal prostheses [14], and biosensors [15].

We developed a 3D bioprinter and applied it to neural cell patterning and culture in single-layer and multilayer collagen hydrogel scaffold. To our knowledge, this is one of the first demonstrations of such method. Recently, direct injection/printing of cells with hydrogel materials has been studied to construct tissues and organs in 3D [16,17]. These direct cell printing methods, such as inkjet printing [5,18–21] or laser printing, have shown limited utility in constructing multilayered cell scaffolds. Through the application of the nebulized crosslinker as a fine mist (diameter $< 2\,\mu m$) to crosslink the dispensed hydrogel, we were able to construct the multilayered cell–hydrogel composite.

Different densities in cell suspension were used for cell printing. A higher cell concentration for neurons was needed to exhibit a degree of cellular connectivity by neurite outgrowth. The density of printed cells in the hydrogel was also adjusted by changing the printing resolution. As shown in Fig. 2a, b, c and d, the spatial distribution of the neural cells was important in subsequent cultures since their connectivity and growth characteristics were affected by cell density in collagen scaffold. Another key component for successful induction of outgrowth of neurites was the concentration of the collagen, whereby active neurite outgrowth was observed in 1.12 mg/ml of collagen precursor without compromising the structural integrity.

Based on the fluorescent images of cultured neurons in a single-layered collagen scaffold on day 15 after printing (Fig. 3a and b), fewer cells were observed in the lowest neuron ring away from the media (Fig. 3e). This observation is contradictory to the previous work by O'Connor *et al.* [9] whereby aggressive neural cell growth and viability was shown after 14 days of culture in thicker collagen scaffold ($> 2.5\,mm$). We conjecture that the reduced cell viability in the lowest hydrogel block might have been attributed to decreased level of perfusion; however, the effects from the inhomogeneity present

during cell printing as well as the altered mechanical property (such as porosity) of the hydrogel block (in which the lower cell layer might have exposed to) cannot be completely ruled out as the contributing sources. Systematic monitoring on the long-term viability and cell growth pattern, including the coculture condition, is warranted, and constitutes a subject for further investigation.

Conclusion

Presented on demand, 3D bio-printing technique will serve as a flexible tool needed for the creation of artificial neural tissue, which enables the examinations both cell-to-cell interactions and the effects of the microcellular environment on neural growth and migration.

Acknowledgements

Authors would like to thank Dr Lai Ding and Mr Daniel Tom of the Harvard NeuroDiscovery Center. They also acknowledge the support from the Focused Ultrasound Surgery Foundation, Gerald J. and Dorothy R. Friedman Foundation and Chung Moon Soul Center for BioInformation and BioElectronics, KAIST.

References

1 Scholl M, Sprossler C, Denyer M, Krause M, Nakajima K, Maelicke A, *et al.* Ordered networks of rat hippocampal neurons attached to silicon oxide surfaces. *J Neurosci Methods* 2000; **104**:65–75.
2 Tooker A, Meng E, Erickson J, Tai YC, Pine J. Biocompatible parylene neurocages. Developing a robust method for live neural network studies. *IEEE Eng Med Biol Mag* 2005; **24**:30–33.
3 Sanjana NE, Fuller SB. A fast flexible ink-jet printing method for patterning dissociated neurons in culture. *J Neurosci Methods* 2004; **136**:151–163.
4 Landers R, Hübner U, Schmelzeisen R, Mülhaupt R. Rapid prototyping of scaffolds derived from thermoreversible hydrogels and tailored for applications in tissue engineering. *Biomaterials* 2002; **23**:4437–4447.
5 Boland T, Xu T, Damon B, Cui X. Application of inkjet printing to tissue engineering. *J Biotechnol* 2006; **1**:910–917.
6 Xu T, Jin J, Gregory C, Hickman JJ, Boland T. Inkjet printing of viable mammalian cells. *Biomaterials* 2005; **26**:93–99.
7 Lee W, Debasitis JC, Lee VK, Lee JH, Fischer K, Edminster K, *et al.* Multi-layered culture of human skin fibroblasts and keratinocytes through three-dimensional freeform fabrication. *Biomaterials* 2009; **30**:1587–1595.
8 Krewson CE, Chung SW, Dai W, Mark SW. Cell aggregation and neurite growth in gels of extracellular matrix molecules. *Biotechnol Bioeng* 1994; **43**:555–562.
9 O'Connor SM, Stenger DA, Shaffer KM, Ma W. Survival and neurite outgrowth of rat cortical neurons in three-dimensional agarose and collagen gel matrices. *Neurosci Lett* 2001; **304**:189–193.
10 Othon CM, Wu X, Anders JJ, Ringeisen BR. Single-cell printing to form 3D lines of olfactory ensheathing cells. *Biomed Mater* 2008; **3**:034101.
11 Gottfried C, Cechin SR, Gonzalez MA, Vaccaro TS, Rodnight R. The influence of the extracellular matrix on the morphology and intracellular pH of cultured astrocytes exposed to media lacking bicarbonate. *Neuroscience* 2003; **121**:553–562.
12 Richards GR, Smith AJ, Parry F, Platts A, Chan GK, Leveridge M, *et al.* A morphology- and kinetics-based cascade for human neural cell high content screening. *Assay Drug Dev Technol* 2006; **4**:143–152.
13 Erickson J, Tooker A, Tai YC, Pine J. Caged neuron MEA: a system for long-term investigation of cultured neural network connectivity. *J Neurosci Methods* 2008; **175**:1–16.
14 Peterman MC, Mehenti NZ, Bilbao KV, Lee CJ, Leng T, Noolandi J, *et al.* The artificial synapse chip: a flexible retinal interface based on directed retinal cell growth and neurotransmitter stimulation. *Artif Organs* 2003; **27**:975–985.
15 Prasad S, Zhang X, Yang M, Ozkan CS, Ozkan M. Neurons as sensors: individual and cascaded chemical sensing. *Biosens Bioelectron* 2004; **19**:1599–1610.
16 Boland T, Mironov V, Gutowska A, Roth EA, Markwald RR. Cell and organ printing 2: fusion of cell aggregates in three-dimensional gels. *Anat Rec A Discov Mol Cell Evol Biol* 2003; **272**:497–502.
17 Sachlos E, Czernuszka JT. Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. *Eur Cell Mater* 2003; **5**:29–39.
18 Wilson WC Jr, Boland T. Cell and organ printing 1: protein and cell printers. *Anat Rec A Discov Mol Cell Evol Biol* 2003; **272**:491–496.
19 Mironov V, Boland T, Trusk T, Forgacs G, Markwald RR. Organ printing: computer-aided jet-based 3D tissue engineering. *Trends Biotechnol* 2003; **21**:157–161.
20 Roth EA, Xu T, Das M, Gregory C, Hickman JJ, Boland T. Inkjet printing for high-throughput cell patterning. *Biomaterials* 2004; **25**: 3707–3715.
21 Xu T, Petridou S, Lee EH, Roth EA, Vyavahare NR, Hickman JJ, *et al.* Construction of high-density bacterial colony arrays and patterns by the ink-jet method. *Biotechnol Bioeng* 2004; **85**:29–33.