ORIGINAL ARTICLE

Control of Retinal Ganglion Cell Positioning and Neurite Growth: Combining 3D Printing with Radial Electrospun Scaffolds

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Retinal ganglion cells (RGCs) are responsible for the transfer of signals from the retina to the brain. As part of the central nervous system, RGCs are unable to regenerate following injury, and implanted cells have limited capacity to orient and integrate *in vivo*. During development, secreted guidance molecules along with signals from extracellular matrix and the vasculature guide cell positioning, for example, around the fovea, and axon outgrowth; however, these changes are temporally regulated and are not the same in the adult. Here, we combine electrospun cell transplantation scaffolds capable of RGC neurite guidance with thermal inkjet 3D cell printing techniques capable of precise positioning of RGCs on the scaffold surface. Optimal printing parameters are developed for viability, electrophysiological function and, neurite pathfinding. Different media, commonly used to promote RGC survival and growth, were tested under varying conditions. When printed in growth media containing both brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), RGCs maintained survival and normal electrophysiological function, and displayed radial axon outgrowth when printed onto electrospun scaffolds. These results demonstrate that 3D printing technology may be combined with complex electrospun surfaces in the design of future retinal models or therapies.

Introduction

RETINAL GANGLION CELLS (RGCs) fail to regenerate and often die following injury or in disease, which has motivated the study of several cell scaffolding and transplantation techniques.^{1–3} Such studies have demonstrated the ability of transplanted RGCs to integrate dendritically into the existing retina. However, the axons of transplanted cells fail to properly orient along existing axon bundles and do not follow soluble or extracellular matrix-based guidance factors to the optic nerve head.¹

Soluble and immobilized guidance factors have been used to direct RGC neurite outgrowth in *in vitro* systems,^{4–6} whereas tissue-engineered scaffolds have been used to create the radial patterning of the rodent retina.⁷ The addition of guidance factor gradients on the surface of tissue-engineered scaffolds enhanced the polarization of axon growth toward the optic nerve head, mimicking the physiology of axon guidance found during early retinal devel-

opment.⁸ These methods, however, have not been able to recreate the more complex organization of the ganglion cell layer (GCL) and retinal nerve fiber layer (RNFL) around the fovea, thus limiting their clinical potential.

The human fovea is characterized by two distinctive features: RGC somas are excluded from the area of the centralis of the fovea and RGC axons are guided around the fovea to reach their intermediate target at the optic nerve head.⁹ Formation of the fovea is mediated through the temporal expression of both soluble and matrix proteins as well as physical forces such as intraocular pressure. These factors induce neural migration and inhibit glial migration and angiogenesis.^{10–12} Methods have recently been developed for the temporal expression of proteins on tissue-engineered scaffolds^{13,14} However, the lack of a vascular system to create the boundary of the fovea and the inability to recreate the full molecular complement of foveal guidance and cell positioning factors highlight the need to explore other methods to recreate the patterning of the GCL.

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Recently 3D printing has been used to recreate the cellular organization of several different tissues, including the bladder,¹⁵ cardiac muscle,¹⁶ and cartilage,¹⁷ as well as models of other tissues such as parts of the brain.¹⁸ Methods for 3D printing of cells and matrix material have variously included colloidal extrusion, laser directed writing, and noncontact jetting.¹⁹ Jetting technologies, previously shown to be compatible with neuronal cells,²⁰ provide a balance between precision and throughput that is appealing for cell placement across macroscopic structures that may be suitable for retinal tissue engineering.

Three-dimensional printing has the potential to specify cell positioning to improve RGC placement in conjunction with a radial electrospun scaffold designed to regulate axon guidance. In this study, we examine the effects of thermal inkjet printing on survival and neurite growth of primary RGCs *in vitro* when printed in different buffers, at different ejection energies, and at different cell densities. Electrophysiological function of RGCs exposed to the stresses of thermal inkjet printing was compared to nonprinted cells. Finally, preliminary patterns were printed to incorporate areas for the fovea onto electrospun scaffolds. We demonstrated the ability to combine these two methods of forming a pattern-organized construct that incorporates cell guidance to the scaffold center.

Materials and Methods

RGC purification

RGCs were purified through a two-step immunopanning process described previously.^{7,21,22} Briefly, retinas were dissected from early postnatal (postnatal days 2–5) Sprague Dawley rats in accordance with protocols approved by the University of California San Diego, Institutional Animal Care and Use Committee (UCSD IACUC) and digested in papain (165 U/L; Worthington Biochemical Corporation, Lakewood, NJ). Retinas were then broken down to a single-cell retinal suspension by mechanical trituration. Macrophage and endothelial cells were depleted through reaction with anti-rat macrophage (Accurate Chemical, Westbury, NY) and RGCs isolated through Thyl1 reactivity.

RGCs were resuspended at varying concentrations in one of the following three media for 3D printing experiments: (1) growth media, a Neurobasal media supplemented with insulin, sodium pyruvate, penicillin/streptomycin, n-acetyl cysteine, triiodothyronine, forskolin, Sato, B27, and brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), growth factors, at previously published concentrations^{21,22}; (2) panning media, Dulbecco's phosphate buffered saline (DPBS) supplemented with bovine serum albumin (BSA; 0.01%) and insulin (5 µg/mL); or (3) DPBS supplemented with fetal bovine serum (30%).

Electrospinning

Radial scaffolds were electrospun as previously described.⁷ Briefly, polylactic acid (PLA, PDL20; Corbion Biomaterials, Lenexa, KS) was dissolved at a concentration of 6.6% in 1,1,1,3,3,3 hexafluoroisopropanol (HFIP; ChemImpex International, Wood Dale, IL). The solution was pumped through a 20-gauge stainless steel needle at a rate of 2 mL/h with the needle charged at 15 kV. A radial collector

consisting of 1.8-cm aluminum foil-covered cup with an 18-gauge copper wire in the center grounded to the same ground as the aluminum foil was used as the target, positioned 12 cm from the needle.

Thermal inkjet printing

RGCs suspended in various media were loaded into an HP TIPS print head (Hewlett-Packard, Corvallis, OR). The HP TIPS thermal inkjet printing system enables fine control of waveform and energy output in the droplet dispensing process and provides for interchangeable print head nozzles. For these experiments, print nozzles were selected with an 80 µm diameter. Print nozzles were initially wetted with forward pressure, after which a backpressure of ~1000 Pa was applied. For cell viability and electrophysiology experiments, an array of eight nozzles were used. A single nozzle was used for cell patterning experiments.

Jetting parameters were selected based on pilot experiments. Dispensing frequency was 50 Hz and pulse width was set to 1 ms. Voltage was initially tested at 25, 27, and 29 V; 27 V was selected for remaining experiments.

For patterning experiments, the print head was suspended over a pair of linear actuators in an XY configuration (Sigma-Koki, Tokyo Japan). Patterns for printing were converted to raster instruction sets from PNG images using MATLAB (MathWorks, Natick, MA). The print head was synchronized with the XY stage such that 10 droplets were dispensed per 1 mm of motion.

After initial patterning experiments, a need to further fix cell position was recognized. To increase media viscosity and prevent cells from flowing away from printed positions, media were blended with the alginate solution to a final concentration of 0.2% alginate.

The initial pattern consisted of a single pass of five parallel lines spaced 0.6 mm from each other. Line numbers 1, 2, 4, and 5 were 10 mm long and line 3, in the center, was only 5 mm long (Fig. 3). The second, more detailed, retinal pattern consisted of a radially spoked circle, 5 mm in diameter, with a 1-mm-diameter void space at the center (Fig. 4).

Live/dead staining

Control RGCs and RGCs were printed in different media, at different ejection voltages, or at different cell concentrations, directly into a 500-µl microcentrifuge tube, resuspended in growth media, and transferred to culture plates where they were cultured for 24 or 72 h. RGCs were then incubated with calcein AM (1:500 of 1 mg/mL dissolved in DMSO; Life Technologies, Carlsbad, CA) and SYTOX Orange Dead Cell Stain (1:500 of 1 mg/mL; Life Technologies) in growth media for 30 min at 37°C and 10% CO₂. Following incubation, a fresh culture medium was added.

Samples were imaged using an Axio Observer Z1 inverted fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany) and categorized as living cells (defined as calcein positive, SYTOX negative), living cells with neurites (visualized with calcein), and dead cells (SYTOX positive). Values for each culture condition were measured for four to five independent experiments with conditions run in triplicate. Data were evaluated for significance using an analysis of variance with *post hoc* testing using Fisher's least significant difference analysis at a significance level of 0.05.

Immunostaining and cellular alignment

Immunostaining and RGC alignment on printed samples were conducted as described previously.⁷ Briefly, scaffolds containing printed cells were fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) diluted in phosphate-buffered saline (PBS). Samples were washed 3× with fresh PBS and then blocked for 30 min with 10% goat serum (Life Technologies) containing 0.2% triton X-100 (EMD Millipore, Billerica, MA) to permeabilize the cell membrane. RGC tubulin was stained using the E7 antibody (mAB, 1:500; Developmental Studies Hybridoma Bank, Iowa City, IA) overnight at 4°C, washed 3× with PBS, and labeled overnight with Alexafluor 488 goat anti-mouse IgG (1:500; Life Technologies).

Mosaic images of whole samples were imaged using an LSM 710 laser scanning confocal microscope containing a 5% overlap used for image reconstruction using Zen Imaging Software (Carl Zeiss AG). RGC neurites were classified morphologically as axons or dendrites according to criteria described previously,²³ with all neurites greater than two cell bodies in length counted manually for alignment $\pm 10^\circ$ with respect to the central point of the scaffold. For cells that contained only a single neurite, it was counted as an axon regardless of morphology once it reached the two cell bodies in length criteria. Significance was analyzed by an unpaired Student's *t*-test at a level of $p < 0.05$.

Electrophysiology

Whole-cell patch clamp recordings were conducted on control and printed RGCs cultured for 48 h on a PDL/laminin substrate using standard wall borosilicate glass patch pipettes (Warner Instruments, Hamden, CT) with tip resistances between 4 and 6 MOhm. Cultured cells were incubated in an external bath of Ames media equilibrated with bubbled 5% CO₂ and current injected using an intracellular pipette solution of 125 mM K-gluconate, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, 10 mM HEPES, 2 mM Na₂-ATP, and 0.5 mM NaGTP and adjusted to pH 7.2 with KOH.

Current clamp recordings were made using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 10 kHz with a low-pass filter (Warner Instruments) and digitized at 5 kHz (Digidata; Molecular Devices). Pipette resistance was compensated by adjusting pipette offset. After break-in, the cells were held at -60 mV and stimulated with 200 ms current pulses. Traces were analyzed using Clampfit (Molecular Devices). Measured values for control and printed cells were evaluated for significance using an unpaired Student's *t*-test at a significance level of $p < 0.05$.

Results

Printing medium significantly affects printed RGC survival and neurite outgrowth

To determine the optimal parameters for cell printing, primary rat RGCs were suspended at a concentration of 250,000 cells/mL. A concern about pH insult to the RGCs, due to inkjet printing in a non-CO₂-controlled environment coupled with the increased surface area of a printed droplet, led us to compare growth media,^{24,25} a neurobasal-based CO₂-buffered media (optimized for RGC culture), to DPBS-based media (which could maintain proper pH throughout

the printing process). In addition, concerns over the need for increased protein buffering to overcome shear forces experienced led us to compare a high-protein (30% FCS in DPBS) and low-protein (0.2% BSA in DPBS) media for RGC printing.

Following 24 h *in vitro*, RGCs were assayed for survival and neurite growth (Fig. 1A), normalizing to RGCs resuspended and plated in growth media but not printed (Fig. 1B). When normalized to control cells, RGCs printed in optimized growth media demonstrated a significantly higher survival and neurite outgrowth when compared to RGCs printed in 30% FCS or 0.2% BSA in DPBS despite the lack of pH buffering.

Thermal print head voltage settings and cell densities do not affect survival or neurite growth

We next determined the effect of ejection voltage, an important parameter for ejection of liquids from a thermal inkjet printer, on RGC survival and neurite outgrowth. The voltage is directly proportionate to the maximum impulse applied to the droplet during ejection. RGCs suspended in growth media were printed at 25, 27, and 29 V (Fig. 1C), a range of voltages derived from the minimum voltage to form a droplet (25 V) through the maximum voltage enabled for the printing system (29 V). While a slight decrease in neurite initiation was observed in RGCs printed at 29 V, no statistical difference was observed at different voltages. Thus, survival and neurite growth were not dependent on ejection voltage within this range, and in subsequent experiments, 27 V was used as the ejection voltage.

Cell density in the bioink was hypothesized to have an effect on viability and neurite outgrowth, while also determining the density of the printed pattern. Cell density may also play a role in inkjet function with medium components or aggregation of cells obstructing the jetting nozzles. To determine the optimal concentration of cells, RGCs were suspended at different concentrations ranging from 2.5×10^5 to 2×10^6 cells/mL of growth media. A cell density of 2×10^6 cells/mL was chosen as the upper practical limit based in the number of RGCs purified and also matched the cell density used in previous studies involving hippocampal cells.²⁰

To detect nozzle obstruction, RGCs were printed into a microcentrifuge tube and then transferred to a culture plate. All living and dead cells were counted and the total number of cells at each printed concentration was normalized to the printed or control samples at the lowest concentration (2.5×10^5 cells/mL) for that day's experiment, to correct for experimental variation. Controls and printed cells increased at the same rate (Fig. 1D), suggesting that no clogging or significant cell loss occurred at higher concentrations. In addition, increased cell density had no effect on RGC survival and neurite growth when compared to control non-printed RGCs after 24 h in culture (Fig. 1E).

Having determined the acute 24-h effects of different conditions, it was necessary to determine if survival and neurite outgrowth were effected at an extended time point. Using the optimized settings of printing at 27 V in growth media at a density of 2.5×10^5 cells/mL, printed and control unprinted cells were cultured for 72 h. Analyzing for survival and for neurite outgrowth, there was no significant difference between controls and printed cells (Fig. 1F).

FIG. 1. Optimal printing promotes RGC survival and neurite outgrowth. Purified RGCs were analyzed using live/dead staining (**A**) for survival with neurite outgrowth (blue arrows), survival without neurite outgrowth (yellow arrows), and dead cells (red arrows) following 24 or 72 h in culture. RGCs printed in different media (**B**), at different ejection voltages (**C**), and at different initial densities (**D**, **E**) were assayed after 24 h in culture. RGCs printed in growth media had an increased survival and increased number of cells projecting neurites when compared to RGCs printed either in FCS or 0.2% BSA ($*p < 0.05$; NS = not significant). No change was observed in survival or neurite outgrowth for RGCs printed at different ejection voltages (**C**) or at different cell densities (**E**). The numbers of printed RGCs increased at the same rate as control cells (**D**) indicating a lack of clogging during the printing process. Using the optimized settings of printing in growth media at 27 kV at a density of 2.5×10^5 RGCs, the cells were further analyzed after 72 h to determine if survival or neurite outgrowth was affected at a later time point. However, no change was observed when compared to control unprinted cells (**F**). RGCs were normalized to control unprinted cells from individual experiments with error bars reporting standard error. Scale bar for (**A**) = 100 μ m. BSA, bovine serum albumin; RGC, retinal ganglion cell. Color images available online at www.liebertpub.com/tea

Thus, bioprinting with these parameters conferred no added toxicity to RGC cultures.

Printed RGCs maintain normal electrophysiological properties

Following selection of optimal printing conditions for RGC survival and neurite outgrowth, we next used whole-cell patch clamp to determine whether the electrophysio-

logical function of RGCs was altered by cell printing at a density of 0.5×10^6 RGCs/mL media. Action potentials of similar scale and threshold were elicited from both control and printed cells on injecting current (Fig. 2A).

Further comparisons of electrophysiological properties, including input resistance, capacitance and action potential characteristics of threshold, peak, half-width, and time-to-peak, were similar between control and printed RGCs (Fig. 2B, $n = 6$ for both groups, with no values significantly

FIG. 2. Printed RGCs fired action potentials and maintained their electrophysiological properties when stimulated. Control and printed RGC electrophysiological properties were analyzed by whole-cell patch clamp with both control and printed cells able to form action potentials following the injection of current (**A**). Whereas printed cells required a larger rheobase current (pA) to induce action potentials, no other significant differences were observed in measured electrophysiological parameters, including the time constant (ms), membrane capacitance (pF), time-to-peak (ms), action potential half width (ms), the peak amplitude (mV), and the threshold potential (–mV) (**B**). * $p < 0.05$, error bars report standard error.

different at the $p < 0.05$ level). However, there was a significant increase in the current required to stimulate action potentials in the printed cells. Thus, although printed RGCs produced a similar response to control RGCs, they required a greater input to produce a response than control cells.

Specific patterns of printed RGCs are retained and increased neurite outgrowth orientation is produced

Having determined the optimum RGC printing conditions, we next determined the physical printing parameters using 2×10^6 RGCs/mL growth media as the bioink and electrospun scaffolds as the recipient matrix. Initially, a simple pattern was created consisting of five lines designed for a single pass of the print head with the middle line half the length of the outside lines (Fig. 3A). However, printed cells were not able to retain this simple cell positioning following the addition of growth media to the printed sample (data not shown). We attributed this to the insufficient ability of the RGCs to bind to the nanofiber scaffold before the addition of media.

To increase RGC adhesion to the scaffolds at the time of printing, we attempted a number of approaches using laminin,

FIG. 3. Printed RGCs maintained patterning. Using a simplified pattern of five parallel lines (**A**), RGCs were printed on electrospun scaffolds with Matrigel in a single pass for each line (**B**). Following the adding of media, RGCs were able to maintain their linear position on the scaffold (**C**). The average line thickness of printed cells was found to be $292 \pm 22 \mu\text{m}$. Scale bar represents 1 mm.

alginate, or Matrigel. RGCs printed on laminin-coated nanofiber scaffolds were permitted 5 min to adhere, yet this did not improve cell pattern retention after 24 h. Alginate hydrogel (0.2%) was then added to the print media to increase the viscosity during the printing process and stabilize the cell positioning. Calcium chloride crosslinking solution (120 mM CaCl_2 , 150 mM NaCl and 25 mM HEPES, Sigma) was added after printing, to crosslink the alginate and entrap cells into their specific position on the scaffold; however, again, the cells were washed to the periphery during the addition of the crosslinking solution. We next attempted scaffolds coated in Matrigel with partial success.

Finally, we embedded electrospun scaffolds in Matrigel before printing in combination with 0.2% alginate. In this system, before printing, Matrigel-electrospun composite scaffolds were incubated in the crosslinking solution and then dried to initiate the crosslinking of the alginate during printing. RGCs printed onto Matrigel-coated scaffolds in combination with the alginate were able to maintain their patterning following the addition of media as evidenced by

samples stained for the nuclear marker DAPI (Fig. 3B, C). In these samples, the average width of RGC printed lines was $292 \pm 22 \mu\text{m}$. Thus, cell positioning onto 3D, Matrigel-coated nanofiber scaffolds was maintained after printing.

Using the physical parameters determined from printing parallel lines, we printed an approximation of the RGC organization in the human retina of a radial pattern containing void areas for the optic nerve head (center of pattern) and the fovea (right of pattern) (Fig. 4A). RGCs incubated with calcein AM for 30 min before printing were visualized following printing onto the scaffold and maintained positioning (Fig. 4B). Finally, we determined whether print-positioned RGCs extended neurites in the radial pattern guided by the scaffold consistent with RNFL patterning. Separate samples were cultured for 3 days, fixed, and their neurites analyzed for alignment with the scaffold fibers.

Neurites were morphologically classified as axons, (long neurites with a constant diameter) or dendrites (neurites larger at the cell body and tapering as they extend away). Radial alignment was recorded for $71.9\% \pm 8.2\%$ of axons and $49.3\% \pm 4.0\%$ of dendrites (Fig. 4C, D), compared to a defined control alignment of 11%. Thus, 3D printing RGCs onto a Matrigel-coated radial scaffold maintained cell positioning in a printed pattern, while also guiding their axons to recreate the organization of the inner retina.

Discussion

The lack of regeneration of RGCs and other CNS neurons following injury and disease has led to the exploration of cellular transplantation therapies. The particular cellular patterning of the human retina, as well as the focal cell loss in asymmetrical degenerations in glaucoma and other diseases of the optic nerve, motivates the need for a method of simultaneously patterning transplanted cells and their growing neurites. Three-dimensional printing has become a valuable tool in the development of transplantable constructs, providing the ability to create complex patterns of both matrix material and cells in multiple layers. This technique would appear particularly well suited to the retina where specific cell types exist in distinct lamina.

Several different CNS neurons have been bioprinted with high viability using thermal inkjet systems, including embryonic cortical neurons,²⁶ embryonic motor neurons,²⁷ and embryonic hippocampal neurons.²⁰ In addition, adult retinal cells have been printed in Neurobasal-based media using a piezoelectric inkjet system with 69% viability.²⁸ We investigated not just viability and spatial patterning but also electrophysiological function and axon patterning of printed cells.

RGC survival was not affected during the printing process, in contrast to a previous study using thermal inkjet printing of hippocampal and cortical neurons.²⁰ This was possibly due to differences in neuronal type or due to media that better buffered against stresses during printing. RGC electrophysiological function was affected, however, requiring an increase in the minimum injected current to produce an action potential. This result likely represents an underlying injury to the printed RGCs. An increase in rheobase current has previously been observed in injury models, even when other electrophysiological properties remained unaffected.²⁹ This loss of sensitivity in the RGCs may be compounded after transplantation into injured retinas.

FIG. 4. Printed RGCs maintained complex positioning and radial neurite patterning on electrospun, hydrogel-embedded nanofiber scaffolds. A more complex pattern (A) was devised to approximate the arrangement of the human retina, including a higher cell density near the optic nerve head and void areas over the fundus (*center*) and at the fovea (*). Following the printing of a single pass for each line, RGCs maintained this pattern (B). Over 3 days in culture, neurites elongated along the scaffold fibers (C). Neurites were classified morphologically as axons or dendrites and counted for alignment with the scaffold fibers. Seventy-two percent of axons and 49% of dendrites were found to extend radially along the scaffold fibers (* $p < 0.05$) (D). Scale bar represents $550 \mu\text{m}$ in (B) and $100 \mu\text{m}$ in (C). Error bars represent standard error. Color images available online at www.liebertpub.com/tea

In models of glaucoma, injured retinas were less able to stimulate inner retinal neurons due to a suppression in bipolar cell signaling.³⁰ Ultimately though, this loss of sensitivity may be recoverable through the chronic application of neurotrophic factors such as CNTF.³¹ It is important to note that the electrophysiological properties assessed were primarily a measure of membrane integrity and the ability of the RGCs to fire action potentials in response to injected current, as RGCs show minimal spontaneous and synaptic activity in purified cultures.³² The ability of these printed cells to respond to synaptic stimulation should be assessed in future studies where the RGCs' synaptic partners, amacrine and bipolar cells, are printed in a second cellular layer.

Because of the limit in precision associated with both the inkjet and hydrogel extrusion methods, we chose to print directly on a biodegradable, radially aligned, electrospun scaffold capable of mimicking the directionality of the nerve fiber layer of the RGCs. Recently, electrospun scaffolds imbedded into hydrogels have been shown to create composites with increased mechanical strength,^{33–35} and hydrogels containing electrospun scaffolds have also been used to guide neural cells in culture.³⁶

In this study, embedding electrospun scaffolds in the laminin-rich hydrogel Matrigel in combination with 0.2% alginate in the media created a suitable substrate to anchor the printed RGCs. Although radial alignment of printed RGC neurites decreased somewhat from 81% on bare scaffolds⁷ to 72% of axons (65% of total neurites) on hydrogel-embedded scaffolds, it still represented a substantial increase over the 11% radial alignment of RGC axons cultured on tissue culture plates⁷ or the 40% radial alignment of RGC neurites when transplanted directly to retinal explants.¹

Hydrogels, such as collagen or gelatin, can be directly electrospun into radial scaffolds for guidance and can absorb the bioink media during the printing process, but these materials become brittle when left unhydrated and without laminin do not promote as much axon outgrowth.³⁷ The use of hydrogel fibers containing a biodegradable polyester core may overcome these disadvantages and increase the mechanical strength of the scaffolds for the printing process.³⁸ Specifically, scaffolds using decellularized extracellular matrix derived from the CNS could include the appropriate substrates for axon outgrowth.³⁹

These data demonstrate aspects of cell positioning and neurite guidance, mimicking certain aspects of the human retina such as the void areas for RGCs at the optic nerve and an increased cell density surrounding the fovea. There are important aspects of GCL patterning, which cannot currently be captured by this method. For example, overall cell density in the human retina is ~ 2100 cells/mm²,⁴⁰ while the current study resulted in a printed density of ~ 30 cells/mm² in a single pass.

Cell density can be increased within the printed pattern by using multiple passes or a stem cell source, which would allow for a higher cell density in the printing solution. Ultimately, replicating the full density of the normal human retina may not be required for a measurable enhancement in visual function. In a crush model, as few as 200 transplanted rod photoreceptors⁴¹ or 131 regenerated axons⁴² have been sufficient to restore light responses to injured retinas.

Another difference between the current data and the human retina derives from the Thy1-based immunopanning strategy

used in this study, which purifies RGCs to >99% purity,^{21,22} but is indiscriminate of different RGC subtypes. There are an estimated thirty RGC subtypes defined by morphology and electrophysiologic response properties.⁴³ Each is tiled with different localizations from the center to the periphery of the retina as well as surrounding the fovea.^{44–46} In addition, it is believed that many of these subtypes are more susceptible to the effects of glaucoma, with cells remodeling and dying off at different rates depending on the RGC subtype.^{47–49}

Specific subtypes of RGCs may self-sort after transplantation, as they preferentially form synapses with specific subtypes of bipolar cells^{50,51} and thereby construct correct retinal circuits, potentially including the proper routing of signals to the brain.⁵² Methods to purify and then print distinct RGC subtypes in their correct patterning may be possible as genetic markers and molecular differences between subtypes are identified.

Conclusions

Recreating the organization of the RGC layer and the guidance of axons to the optic nerve remains a challenge to transplantation of RGCs in degenerative diseases. Here we demonstrated a novel approach to couple the positioning of RGCs using 3D cell printing with the guidance of printed RGC axons using radial electrospun scaffolds embedded in hydrogel matrices, mimicking the cellular organization of the retina. This method maintained cell survival, neurite outgrowth, and functional electrophysiological properties of the printed cells. Together, these data represent a step forward in the development of retinal and neural tissue engineering, combining 3D positioning with axon guidance to recreate complex tissue.

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Author Contributions

K.E.K., S.P.G., J.L.G., and D.D.D. designed the research. K.E.K., S.P.G., E.W.D., P.V., and M.F.M. performed research. K.E.K., S.P.G., E.W.D., P.V., J.L.G., and D.D.D. analyzed data. K.E.K., S.P.G., E.W.D., J.L.G., and D.D.D. wrote the article.

Disclosure Statement

No competing financial interests exist.

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