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A bilayer photoreceptor-retinal tissue model with gradient cell density design: a study of microvalve-based bioprinting

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Abstract: ARPE-19 and Y79 cells were precisely and effectively delivered to form an *in vitro* retinal tissue model via 3D cell bioprinting technology. The samples were characterized by cell viability assay, hematoxylin and eosin (HE) and immunofluorescent staining, scanning electrical microscopy (SEM) and confocal microscopy etc. The bioprinted ARPE-19 cells formed a high-quality cell monolayer in 14 days. Manually seeded ARPE-19 cells were poorly controlled during and after cell seeding, and they aggregated to form uneven cell layer. The Y79 cells were subsequently bioprinted on the ARPE-19 cell monolayer to form two distinctive patterns. The microvalve-based bioprinting is efficient and accurate to build the *in vitro* tissue models with the potential to provide similar pathological responses and mechanism to human diseases, to mimic the phenotypic endpoints that are comparable to clinical studies, and to provide a realistic prediction of clinical efficacy.

Keywords: bioprinting, retina, tissue model, age-related macular degeneration (AMD), retinoblastoma

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1 Introduction

Experimental animal plays a significant role in current scientific and medical research and assists scientists to progressively understand various diseases. Some novel medicines and treatments are developed by the advance of animal research, for example, to investigate the development and spread of retinal tumor(Tschulakow, Schraermeyer et al. 2016). However, the retinal models in primate and rodent may take months to develop designed symptoms (Lamba, Gust et al. 2009, Di Marco, Di Paolo et al. 2014, Katt, Placone et al. 2016, Shirai and Mandai 2016). The use of animals in research is controversial, and the animals suffer in both physical and psychological ways. Strict regulations are continuously implemented over animal trials. Replacement, Reduction, and Refinement (3Rs) have been proposed and considered as guidelines for animal trials in academic and industrial research. *In vitro* three-dimensional (3D) tissue models mimic the cell arrangements in native tissues and organs, meanwhile, provide us effective and productive tools for disease research and drug evaluation (Katt, Placone et al. 2016, Lee, Koo et al. 2017). Therefore, an *in vitro* retinal tissue model is meaningful for investigation of disease mechanism and regenerative strategies, overcoming current limitations. 3D bioprinting is invented to generate organized and biomimetic 3D cell constructs for biomedical applications with improved accuracy and efficiency(Chee Kai Chua 2014), and this technology is used to create bone, blood vessel, heart, skin models in complex structures(Lee and Yeong 2016, Ng, Wang et al. 2016, Shao, Ke et al. 2017, Zhu, Qu et al. 2017). Ocular tissue bioprinting currently faces limitations(Huang and Zhang 2014), only rat retinal cells are bioprinted, and their viability after bioprinting is reported (Barbara, Wen-Kai et al. 2014). A retinal tissue model with complex 3D cellular structure is useful for drug screening and rehabilitative investigation, for example, development of treatment methods for age-related macular degeneration (AMD) and retinoblastoma.

Retinal pigment epithelial (RPE) plays a significant role in retinal photoreceptor homeostasis and actively maintains the overall health of retina (Dunn, Aotaki-Keen et al. 1996, Klimanskaya 2006). Any pathological changes of the RPE will have a devastating effect on vision. Although much information of RPE has been obtained from *in vivo* studies, the isolation, culture, and expansion of mammalian RPE are difficult, for example, losing phenotypic characteristics (Turowski, Adamson et al. 2004). ARPE-19 is developed to simplify *in vitro* RPE cell biological experiments (Turowski, Adamson et al. 2004). The ARPE-19 cell line produces appropriate extracellular matrix *in vitro*, and more importantly, the cell line can develop phenotypic characteristics in close relationship with human RPE *in vivo* (Turowski, Adamson et al. 2004). A monolayer of ARPE-19 cells is key for investigation of polarized viral budding, protein targeting and retinoid metabolism (Dunn, Aotaki-Keen et al. 1996, Dunn, Marmorstein et al. 1998). Optimization of manual ARPE-19 cell seeding density to achieve a high-quality cell layer is challenging (Dunn, Aotaki-Keen et al. 1996, Dunn, Marmorstein et al. 1998, Lu, Lee et al. 2007, Thumann, Viethen et al. 2009, Thomson, Treharne et al. 2011, Shadforth, George et al. 2012). Controlling ARPE-19 cell seeding patterns using paraffin inserts and soft lithography are also reported (Kane, Takayama et al. 1999, Lu, Kam et al. 1999, Javaherian, O'Donnell et al. 2011, Vargis, Peterson et al. 2014). Y79 cell line is derived from human retinoblastoma (Reid, Albert et al. 1974), and it grows in suspension forming clusters, chains, and rings. The Y79 cells can differentiate *in vitro*, depending on culture and processing methods (Kyritsis, Tsokos et al. 1987), for example, the addition of succinylated concanavalin A (Seigel and Notter 1993) and activin (Kanno, Kashiwagi et al. 2009) may induce differentiation of Y79 towards rod photoreceptor cells. Moreover, neuronal and glial differentiation of Y79 cells *in vitro* can be evaluated through observation of cellular morphology (Kyritsis, Tsokos et al. 1984, Chader 1987, Sheffield, Stauber et al. 1990, Seigel and Notter 1993, Vento, Giuliano et al. 1997). To build ARPE-19 monolayer and acquire ARPE-19 and Y79 cell seeding patterns utilizing microvalve-based bioprinting are rarely reported. The microvalve based bioprinting technology can accurately create tissue models with a high degree of throughput (Ng, Lee et al. 2017). In this paper, a 3D retinal tissue model (Figure 1) composed of ARPE-19 and Y79 cells is bioprinted, and the bioprinted construct is meaningful for biomedical applications, for example, disease research and discovery of treatment options.

2 Materials and Methods

2.1 Cell culture

The human retinal pigmented epithelial cell line, ARPE-19 (CRL-2302; ATCC) and the human retinoblastoma cell line, Y79 (HTB-18, ATCC) were maintained in DMEM:F12 (ATCC) and RPMI 1640 (ATCC) media at 37 °C with 5% CO₂ respectively, and the media was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics.

2.2 ARPE-19 Cell density optimization

To investigate the best ARPE-19 cell seeding density to form monolayer, 10⁴ (Density 1), 5×10⁴ (Density 2), 10⁶ (Density 3) and 5×10⁶ (Density 4) ARPE-19 cells were seeded in each well of 12 well-plates (Corning, Approx. Growth Area: 3.8 cm²) respectively, the cell viabilities were constantly monitored by PrestoBlue (Thermo Fisher Scientific) assay according to vendor's protocol, and test media (1ml) consisted of PrestoBlue (10%), FBS (5%) was added and incubated in each well for 30 mins; the cells in each well were also stained by ActinGreen 488 ReadyProbes and NucBlue Live ReadyProbes reagents (Thermo Fisher Scientific) and observed under inverted microscope (Zeiss).

2.3 Cell viability evaluation

To investigate the cell viabilities during and after bioprinting, the ARPE-19 and Y79 cells were treated under 1, 2, 3 and 4 bars of pneumatic pressure for 5mins respectively, and they were bioprinted through a microvalve nozzle (pneumatic pressure: 0.25bar, opening time: 0.5ms and frequency: 1000 Hz). The samples were collected in 10 ml tubes and reconstituted to 5×10⁴ cells/ml individually, and then 1 ml of ARPE-19 cell suspension was seeded in a well of 24 well-plate (Corning), while 1 ml of Y79 cells were aliquoted in a 10ml tube respectively. The cell viabilities were monitored by PrestoBlue (Thermo Fisher Scientific) assay, test media (1ml) was composed of PrestoBlue (10%), FBS (5%). Control groups were cells without any treatment.

2.4 Microvalve-based bioprinting

RegenHU bioprinter (Switzerland) was used to print the retinal tissue model. The ARPE-19 cells were washed in phosphate buffered saline (GE Hyclone), and then they were trypsinized by TrypLE (5ml, Thermo Fisher Scientific) upon confluence and neutralized by 5 ml DMEM: F12 media. The cells were counted, centrifuged and re-suspended in the media (10^6 cells/ml). The Y79 cells in suspension were also counted, centrifuged and re-suspended in RPMI-1640 media (10^6 cells/ml). The ARPE-19 and Y79 cells in culture media were loaded into cartridges that were kept under constant agitation before bioprinting. The bioprinting tool paths for ARPE-19 and Y79 can be seen in Figure 1. The first layer: the ARPE-19 cells were printed on glass substrates within a circular shape (diameter: 20mm, average cell density: 2786 ± 492 cells/cm², approximately 111 ± 20 cells/droplet) and cultured for 2 weeks. The second layer: Y79 cells were printed on ARPE-19 monolayer (approximately 159 ± 6 cells/droplet, diameter: 20mm) with two distinctive patterns; i) a high average density at periphery (OH), average cell density allocation: 4188 ± 153 cells/cm² at inner circle and 10296 ± 376 cells/cm² at annulus; ii) a high average density at the central area (CH), average cell density allocation: 13787 ± 503 cells/cm² at inner circle and 4479 ± 164 cells/cm² at annulus. The samples were cocultured for another two weeks, and fixed in 4% PBS buffered paraformaldehyde at day 1, 7 and 14 respectively for further analysis. The singular ARPE-19 and Y79 cell layers were also printed on the glass substrates separately for cell counting, calculation of seeding densities and observation. The same number of ARPE-19 cells were manually seeded on the substrates within a 20mm circular shape (comparable to the bioprinting-surface area), and the cells were fixed in PBS buffered paraformaldehyde (1ml) at day 1, 7 and 14 respectively as controls.

2.5 Cell staining, bright field, fluorescent and scanning electrical microscopy observation

The manually seeded ARPE-19 cell layer, bioprinted ARPE-19 monolayer, and cocultured samples were stained with hematoxylin/eosin (HE, Sigma-Aldrich), ZO-1 antibody (ZO1-1A12, FITC labeled, Thermo Fisher Scientific), Claudin-1 antibody (2H10D10, Alexa Fluor 555 labeled, Thermo Fisher Scientific) and ActinGreen 488 (Thermo Fisher Scientific) respectively. The nuclei were counterstained with DAPI (Nucblue Live ReadyProbes reagents, Thermo Fisher Scientific) for fluorescent observation. The HE-stained samples were scanned by Live Cell Observer (Zeiss). The fluorescent samples were observed under an inverted microscope (Zeiss) and Laser Scanning Microscope (Zeiss LSM 710). For scanning electron microscope (SEM, JEOL) observation, the fixed samples at day 1, 7 and 14 were dehydrated in 30%, 50%, 75%, 95% and 100% ethanol gradually before critical point drying, and the samples were sputter coated with gold for SEM observation. ImageJ (1.51j) was used to analyze the scanning images of HE stained samples (the distribution of Y79 cells on ARPE-19 monolayer at day 14): the image type was changed to 8 bits and adjusted the threshold to properly exclude the ARPE-19 monolayer, and then measured the integrated intensities of Y79 cells at the inner circle (diameter: 10mm) and the annulus (10 mm > diameter > 5 mm) in both OH and CH groups respectively.

2.6 Statistical analysis

All data were presented as mean $\pm$ the standard deviation ($n \geq 3$). Statistical analysis was implemented by Mann–Whitney U tests and multiple comparisons using Kruskal–Wallis one-way analysis of variance tests, using SPSS Statistics version 19.0, and $p < 0.05$ and 0.01 were statistically significant.

3 Results

3.1 Assessment of ARPE-19 cell seeding density, cell viability after microvalve-based bioprinting

Initial manual cell seeding density demonstrated significant effects on the quality of cell layers. There were cell clusters in four individual cell seeding densities (Density 1: 10^4 cells/well, Density 2: 5×10^4 cells/well, Density 3: 10^6 cells/well and Density 4: 5×10^6 cells/well) at day 1 in Figure S1 a, b, c and d. The amount and size of cell clusters and aggregations increased with the higher cell seeding densities, and multiple cell layers were easy to be observed at day 14. The lowest cell seeding density (10^4 cell/well, 2631 cells/cm²) of ARPE-19 corresponded to the best cell distribution over 14 days (Figure S1 a, a1 and a2). In Figure 2 a, the higher cell seeding density brought the higher values of initial cell viability, and the values dropped gradually at day 7 and day 14. The gradually increased pneumatic pressure did not reduce cell viability over 14 days (Figure 2 b). The development of cell morphology in bioprinting and manual seeding (control) groups was comparable, and most of the cells attached to the substrate and proliferated to confluence at day 14 (Figure 2, a1-a3 and b1-b3).

3.2 Evaluation of ARPE-19 cellular morphology and cell-cell interactions

The overall ARPE-19 cell expansion patterns in bioprinting and control groups were shown in Figure 3 a-f, the cells in bioprinting samples were properly organized to achieve improved proliferation and expansion, and finally to form cell monolayer. While, the manually seeded cells unevenly distributed in control group at day 1, and they accumulated and formed the smaller cell layer in 14 days. The bioprinted cells formed a high-quality cell monolayer with strong and clear ZO-1 and Claudin-1 expressions in cellular interactions, while the cells in control group formed clusters and packed together with the disorder and weak ZO-1 and Claudin-1 expressions (Figure 3 c1-f3).

3.3 Y79 cell microvalve-based bioprinting, culture, and characterization

Y79 cells showed high volume ratio of nucleus to the cytoplasm (Figure 4 a), and their cell viability was not compromised in several bioprinting conditions with increased pneumatic pressures (Figure 4 b). The two printing patterns of Y79 cells on ARPE-19 cell monolayer were bioprinted on coverslips could be seen in Figure 4 c and d. It was easy to observe that the higher average Y79 cell seeding density at the periphery in OH group, while the higher average Y79 cells seeding density at the central area could be observed in CH group. The cell proliferation and morphological changes could be observed in SEM images at day 1, 7 and 14 respectively (Figure 5 a-c). The Y79 cells changed from low to the high aspect ratio (cells turn into flat morphology) with increased cellular attachments. In Figure 5 d, e and f, the ARPE-19 cells maintained the high-quality monolayer with strong and clear ZO-1 and Claudin-1 expressions, meanwhile, the Y79 cells maintained the high-volume ratio of nucleus to cytoplasm according to F-actin staining. The ZO-1 and Claudin-1 staining could also be observed at the interactions between the Y79 and ARPE-19 cells. HE staining of the cocultured samples at day 14 in both groups unveiled that the Y79 cells attached on the ARPE-19 cells, and proliferated over 14 days (Figure 6 a and b). Subsequently, the data analysis (integrated intensity) proved that the Y79 cells maintained the higher average cell density at periphery and center in OH and CH groups over 14 days respectively, as can be seen in Figure 6 a1, a2, b1 and b2. The morphology of Y79 cells on ARPE-19 cell monolayer can be observed in confocal and SEM images (Figure S2), Y79 cells maintained a large volume of cell nuclei while attached to the ARPE-19 monolayer, and cellular interactions could be observed.

4 Discussions

To use microvalve-based bioprinting technology to prepare retinal tissue model has several advantages: i) high throughput: the native tissue structure and cellular interactions can be rapidly reproduced to investigate cell functions, while manual cell seeding) is time consuming with low accuracy(Hamilton, Foss et al. 2007, Wisniewska-Kruk, Hoebe et al. 2012); ii) accuracy and efficiency: the process is well controlled to maximally reduce manpower and exclude man-made errors, and this method can be applied to produce retina on a chip device with improved producibility(Chung, Lee et al. 2017, Yeste, Garcia-Ramirez et al. 2017) ; iii) alternatives to animal models: *in vitro* human tissue models may significantly improve accuracy, efficiency, reproducibility and therapeutic translatability of animal-free experiments, as well as resolve cruelty and ethical concerns of animal testing. Preparation of the blood-retinal-barrier models using animal tissue are also challenging (Steuer, Jaworski et al. 2004). The common issue for application of animal models is the credibility of the single model or collection of models, and to bridge the gap between human and experimental animal models is difficult (McGonigle and Ruggeri 2014). The visual systems in different animals are unique in terms of sizes, structures, refractive properties, density and distribution of photoreceptors(Blanch, Ahmed et al. 2012). For example, an ideal AMD model would recapitulate the histological and functional changes, and allow fast and efficient disease development(Pennesi, Neuringer et al. 2012). Meanwhile, investigation of origin of retinoblastoma in patients is restricted by small sample size and inadequate animal models. (Nair, Kaliki et al. 2013). The microvalve-based bioprinting can assist us to build an *in vitro* tissue model with significant advantages. Human cells/cell lines can be bioprinted directly to obtain tissue models with similar genetic basis and native anatomy. Subsequently, these models may be employed to investigate clinical efficacy of drugs and treatment methods.

The automated bioprinter effectively delivers cells and biomolecules to create complex micro-tissues and micro-organs, which is helpful to investigate disease development, drug metabolism and tissue transplantation etc. (Li, Chen et al. 2016, Zhu, Ma et al. 2016). In this article, ARPE-19 and Y79 cells are seeded *via* microvalve-based bioprinting to achieve homogeneous cell seeding and tunable cell patterning respectively. In manual cell seeding methods, even at the minimum cell seeding density, the cells float and aggregate together, finally form clusters. Thus, manual cell seeding is not competitive enough for production of a high-quality cell monolayer. A cell layer containing multiple cell clusters and aggregations may form instantly at the highest cell seeding density (Density 4: 131579 cells/ cm²). The other two intermediate cell seeding densities lead to uneven cell layer at day 1 (Density 2 and 3). The ARPE-19 seeding at density 2, 3 and 4 form overwhelmed cell layers within one week, and over-proliferate in two weeks. The lowest manual cell seeding density achieves the ARPE-19 cell layer at its highest quality in the two weeks, however, a few cell clusters can still be observed. Furthermore, the lowest ARPE-19 cell seeding density brings ascending cell viabilities over 14 days. In contrast, the other three cell seeding densities lead to overcrowded cells and multiple cell layers that may deteriorate their own living conditions, and their cell viabilities decrease within two weeks. Therefore, 2786±492 cells/cm² (comparable to the manual cell seeding density 1) is employed for the microvalve-based bioprinting of ARPE-19 cells .

The microvalve bioprinting strategy is first developed in 2007 to deliver live cells (Demirci and Montesano 2007), and it can produce drop-on-demand cell deposition by swift actuation of the microvalve (Ng, Yeong et al. 2017). Utilization of the microvalve-based bioprinting manner can achieve high-throughput and precise cell seeding, however, the cells will also afford the extra procedures during bioprinting, including pneumatic pressure, cell sedimentation, opening and closing a small valve(Ferris, Gilmore et al. 2013). The cell viabilities of ARPE-19 and Y79 under several bioprinting parameters are carefully evaluated, including modifications of pneumatic pressure from 1 to 4 bars. Conclusively, the microvalve-based bioprinting process shows no acute and systemic cytotoxicity to the cells. It is also worth to point out that the cell sedimentation happens during the bioprinting process, the larger cells deposit faster (ARPE-19) than that of the relatively smaller cells

(Y79), thus, the standard deviation of cell count per ARPE-19 droplet shows a wider range of values. Countermeasures are applied to alleviate the cell sedimentation effects, including constant vibration of the cell loaded cartridge and frequently flushing the cell laden culture medium. The morphological studies of ARPE-19 cells after manual seeding and bioprinting can be observed in SEM images . The cells in both groups maintain normal and similar morphologies, and cell elongation can be observed in bioprinting group, while cell stacks can be identified in manual seeding group. Furthermore, the differences of cell proliferative patterns in the bioprinting and manual cell seeding groups can be overviewed. The cells in bioprinting group are initially seeded in droplet formation, then the cells gradually proliferated and slowly occupied the gaps among each droplet within the printing area, subsequently, the cells expand outwardly at week 2. The manually seeded cells randomly attach on the expected cell seeding area, and the cells float to the central area due to surface tension. Thus, a relatively smaller seeding area can be observed at day 1 (compared to microvalve-based bioprinting), and cell clusters and aggregations formed at day 7 and 14 respectively. Bioprinting and manual cell seeding groups at day 14 are stained by F-actin, ZO-1 and Claudin-1 reagents, stacked cell nuclei in manual seeding group are frequently spotted, while the ARPE-19 cells in bioprinting group form and maintain high-quality monolayer. This conclusion is in the line with a report to build an *in vitro* 3D bioprinted air-blood barrier by Horváth et.al (Horváth, Umehara et al. 2015). The clear ZO-1 and Claudin-1 staining can be observed lining among the cellular interactions in a highly continuous fashion in the microvalve-based bioprinting group, comparing to the blur and scattered staining in manual cell seeding group. Such enhanced distribution of these two proteins suggests that orderly ARPE-19 cell seeding promotes the formation of cell-cell junctions. The intercellular tight junctions are structurally composed of anastomosing strands connecting neighboring cells with semi-permeable and semi-selective seals (Chiba, Osanai et al. 2008), and the RPE monolayer is for proper function of the blood-retina barrier and sensory retina cells, regulating the ionic composition in the sub-retinal space (Dunn, AotakiKeen et al. 1996, Rizzolo 2014). The intact and mature RPE cell monolayer is mitotically quiescent under physiological condition (Chiba 2014, Wang and Yan 2014). The early formed cell clusters and cell layers after manual cell seeding may have the slower metabolism than that of the cells at the edge, therefore, the metabolic discrepancy may delay and even prevent the

formation of the high-quality ARPE-19 cell monolayer. Interestingly, the homogeneously bioprinted ARPE-19 cells at each droplet maintain their proliferative tendency in a parallel fashion until the formation of an excellent cell monolayer.

The microvalve-based bioprinting has demonstrated the inherited advantages to create the cell monolayer. More interestingly, the same technology can be further applied to build Y79 cell patterns on top of the existing cell monolayer (ARPE-19). The same results are time consuming and challenging for manual cell seeding to achieve. The Y79 cells are cultured in suspension with spherical morphology, and then the cells are printed on the ARPE-19 monolayer with two distinctive patterns respectively. The Y-79 cells attach on the ARPE-19 monolayer at day 1 after bioprinting, and then they proliferate with morphological changes to flat cell type at day 7 and 14 progressively, and this process does not involve poly-D-lysine and polyethyleneimine coating. In confocal images, the Y79 cells attach to underneath ARPE-19 cell monolayer, and they maintain high volume ratio of nuclei to the cytoplasm after 14 days' coculture. It is worth to mention that the clear ZO-1 and Claudin-1 staining can still be observed in the cell-cell interactions, thus the addition of the Y79 cells may not affect the normal formation of tight junctions at the ARPE-19 cell borders. The close interactions of between Y79 and ARPE-19 cells can be clearly observed at respective side views of confocal images. Furthermore, the cellular interactions of Y79 and ARPE-19 cells can be monitored, and the cytoplasmic projection of Y79 cells can be clearly observed, attaching on the ARPE-19 cell monolayer (Figure S2 b, yellow arrows). The projection may provide major roles for stabilizing the Y79 cells during the 14 days' coculture. In details, the average Y79 cell printing densities are 4188 ± 153 cells/cm² at the central area and 10296 ± 376 cells/cm² at the periphery in OH group, while 13787 ± 503 cells/cm² at the central area and 4479 ± 164 cells/cm² at the periphery in CH group respectively at day 1. The proliferation of Y79 cells maintains their high average intensity at the periphery in OH group and high average intensity at the central area in CH group in 14 days according to microscopic observation and ImageJ analysis. This further proves that the cellular interactions between Y79 and ARPE-19 cells, and the cytoplasmic projection is critical to preserve the density difference of Y79 cells during the coculture. The Y79 cells express both cone- and rod-

specific antigens(Bogenmann, Lochrie et al. 1988, Di Polo and Farber 1995, Wiechmann 1996), and Messmer *et al.* report that numerous fresh retinoblastoma tumor cells could differentiate to the photoreceptor, neuronal and glial cell lines(Messmer, Font et al. 1985). Therefore, the Y79 cell line is a useful candidate for the study of disease mechanism, development and possible treatment options of sensory retina malfunction (Tan, Ding et al. 2004). Meanwhile, the inherited density and distribution of human cone and rod photoreceptor cells are different from foveolar to the retinal periphery, and the cone concentration peaks highly at the foveola while rod density is peaked at a distance of 5-6 mm from the foveola (Jonas, Schneider et al. 1992). Thus, both the Y79 cell printing strategies including OH and CH patterns are considered as a consolidated bioprinting plan for stimulation of the disease development, or distribution of photoreceptor cells.

A major trend in regenerative medicine requires a novel and more realistic approach *via* 3D tissue model, such approach is to deeply understand disease mechanism without using animals, finally to evolve treatment options (Francis and Abramson 2015). In nutshell, the ARPE-19 cells actively retain numerous RPE cell characteristics, including functional tight junctions (Dunn, AotakiKeen et al. 1996, Dunn, Marmorstein et al. 1998), the Y79 cells may originate from early retinoblasts, arising before optic cup separation to inner and outer layers that can differentiate into either photoreceptor or glial cells, and the cell line expresses a variety of mature markers representing several retinal cell types (Green, Meek et al. 1979, Chader 1987, Cassidy LL 2012). The microvalve-based bioprinting of retinal tissue model is potentially useful for drug screening, studying retinal cell interactions and the effects of different molecules on retinal regeneration(Maenpaa, Toimela et al. 2004). This is probably the first time that the ARPE-19 and Y79 cells are bioprinted coherently to build the retinal model, and the microvalve-based bioprinting strategies invented in this article will bring more knowledge regarding creation of tissue models for retinal research, and the methodologies can also be applied to create other tissue models.

5 Conclusion

The ARPE-19 and Y79 cells were respectively delivered to discrete places with high efficiency and precision *via* microvalve-based bioprinting, creating a complex retinal tissue model with the capability to simulate key aspects of native/diseased tissue compositions in terms of cell distribution and cell density. More importantly, the high-throughput process brings great opportunities to build the *in vitro* tissue models that are physiologically and pathologically comparable to the native/diseased tissue in animal models. The development of the *in vitro* tissue models may reduce the needs of animal trials, provide clinically translatable data for investigation of disease mechanism, screening treatment options and improving tissue regenerative strategies.

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Supporting Information

Supplementary data is available.

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Figure 1. The diagram of bioprinting process from a target organ (eye) to a bioprinted construct

Figure 2. Cell viability of ARPE-19 at four seeding densities respectively (a, Density 1: 2631 cells/cm², Density 2: 13158 cells/cm²; 26316 cells/cm² and 131579 cells/cm²); the ARPE-19 cell viability under different bioprinting conditions (b); SEM images of manually seeded (a1, a2 and a3) and bioprinted ARPE-19 cells (b1, b2 and b3) at day 1, 7 and 14 respectively; NS: $p > 0.05$ and * $p < 0.05$

Figure 3. Bioprinted ARPE-19 cells (a, b and c) and corresponding manually seeded cells (d, e and f): HE staining at day 1, day7 and day 14 respectively, scale bar: 5mm ; Confocal images of ARPE-19 cell layers at day 14: bioprinting (c1, c2 and c3) and manual cell seeding (f1, f2 and f3); F-actin cytoskeleton immunofluorescence staining (c1 and f1), Zonula occludens-1 (ZO-1, c2 and f2) in green, Claudin-1 (c3 and f3) in red and nuclei in grey, the x-y projections of single optical sections are presented in central images with respective side views in x-z and y-z (up and right); scale bar: 50µm

Figure 4. Fluorescent image of Y79 (a, F-actin: green, nuclei: grey); the Y79 cell viability under different bioprinting conditions(b); scanning images of two Y79 bioprinting patterns (HE staining) at day 1: average cell density 4188 ± 153 cells/cm² at inner circle and 10296 ± 376 cells/cm² at annulus (c), 13787 ± 503 cells/cm² at inner circle and 4479 ± 164 cells/cm² at annulus (d); diameters: small circle (10 mm) and large circle (20 mm); scale bar: 20 μ m (a) and 5mm (c and d).

Figure 5. SEM images of biprinted Y79 on RPE cell monolayer at day 1, day 7 and day 14 (a, b and c); confocal image of biprinted Y79 cells on ARPE-19 cells at day 14: F-actin cytoskeleton staining (d) and Zonula occludens-1 staining (ZO-1, e) in green, Claudin-1 (f) in red and nuclei in grey; the x-y projections of single optical sections are presented in central images with respective side views in x-z and y-z (up and right); scale bar: 10μm (SEM images) and 50μm (confocal images)

Figure 6. HE staining of the bioprinted Y79 cells with 2 distinctive patterns on ARPE-19 cell monolayer at day 14: high density at periphery (OH, a) and high density at the central area (CH, b); data analysis (integrated intensity) of bioprinted Y79 proliferation after 14 days, high density at periphery (OH, a1 and a2) and high density at the central area (CH, b1 and b2); A: area of the inner circle, and B: area of the annulus; diameters: small circle (10 mm) and large circle (20 mm); scale bar: 5mm; ** $p < 0.01$