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Research article

Development of an automated chip culture system with integrated on-line monitoring for maturation culture of retinal pigment epithelial cells

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Supplementary Information

Supplementary Methods

RPE cells and hiPSC-derived RPE cells

We used primary RPE cells, which were positive for anti-ZO-1 and anti-Pan-cytokeratin staining, and negative for anti-fibroblast staining (Lonza, Walkersville, MD), as a source for the study of RPE phenotype in this system. In addition, based on previous reports that human iPSC can be successfully generated RPE cells through small-molecules [1,2], we performed the RPE differentiation from iPSCs and maturation culture. The protocol for RPE differentiation and cultivation was based on Okada et al., who reported the RPE differentiation from human iPSCs. They

described that the treatment induces hexagonal pigmented cells that express RPE65 and CRALBP, form ZO-1-positive tight junctions and exhibit phagocytic functions. Subsequent treatment with retinoic acid and taurine induces photoreceptors that express recoverin, rhodopsin and genes involved in phototransduction. Both three-factor (OCT3/4, SOX2 and KLF4) and four-factor (OCT3/4, SOX2, KLF4 and MYC) human iPSCs could be successfully differentiated into retinal cells by small-molecule induction.

hiPSCs maintenance and differentiation to RPE cells

The protocol for RPE differentiation and cultivation was based on Okada et al. [1,2] with minor modifications. The hiPSC line Tic (provided by JCRB1331, JCRB Cell Bank) was routinely maintained on mitomycin C-treated SNL76/7 cells (European Collection of Cell Cultures, Salisbury, UK) in commercially available medium (ReproStem, ReproCELL) supplemented with 5 ng/mL basic fibroblast growth factor. For retinal differentiation, hiPSC colonies were treated with Y-27632 (10 μM; Wako) for 1 h, and dissociated into clumps with 0.25% trypsin and 0.1 mg/ml collagenase IV in phosphate buffered saline (PBS) containing 1 mM $CaCl_2$ and 20% knockout serum replacement (KSR; Invitrogen). Feeders were removed by incubation of the hiPSC suspension on a gelatin-coated surface for 1 h. hiPSC clumps were incubated on non-adhesive dish (Nunc) in DMEM/F12 supplemented with 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 20% KSR for 3 days, in 20% KSR-containing differentiation medium (GMEM, 0.1 mM non-essential amino acids, 1 mM pyruvate, and 0.1 mM 2-mercaptoethanol; all obtained from Invitrogen) for 3 days, then in 15% KSR-containing differentiation medium for 9 days, and finally in 10% KSR-containing differentiation medium for 6 days. Y-27632 (10 μM) was added for the first 15 days of suspension culture. CKI-7 (5 μM) and SB-431542 (5 μM) were added to the medium for 21 days during the suspension culture. The medium was changed every 3 days. Induced RPE colonies were picked up manually and cultured on a laminin-coated surface in DMEM/F12 supplemented with B27 (Invitrogen) and 10 ng/mL bFGF. The laminin-coated surface was prepared according to protocols described previously [1,2], immediately preceding the start of culture. A 50 μg/ml solution (laminin-1; Sigma-Aldrich) in PBS was introduced into the culture vessel (surface coverage: 10 $\mu g/cm^2$) and incubated for 2 h at 37 ℃, followed by washing with PBS prior to cell seeding. When the culture reached confluence, cells were dispersed with trypsin-EDTA and reseeded.

Maturation culture of hiPSC-derived RPE cells

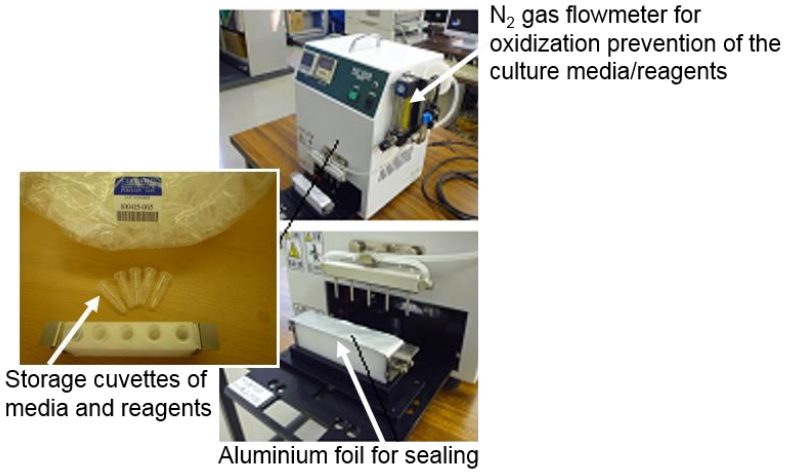
Maturation culturing was performed on a laminin-coated surface in 48-well plates (culture area in each vessel was 0.95 cm^2; Corning Costar), and the viable cells were seeded at 5.0×10^4 cells/cm^2. Images were acquired approximately every 2 days for 36 days using an image analyser (IN Cell Analyzer 2000; GE Healthcare) with a 4× objective lens.

Characterisation of RPE cells

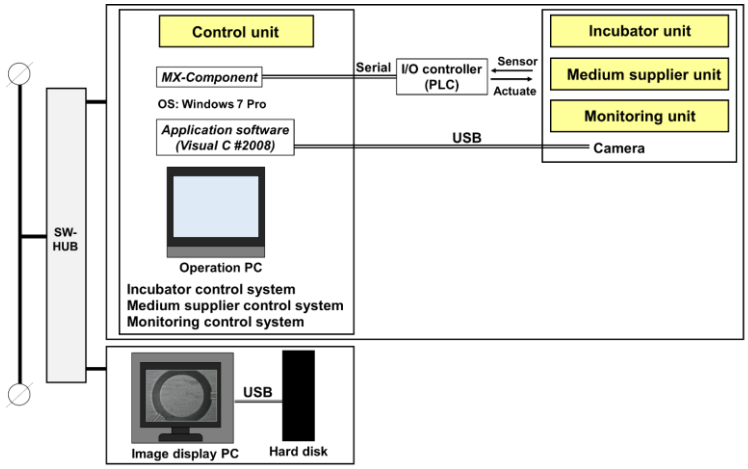
Cells were fixed with 4% w/w paraformaldehyde in PBS into the cell culture chambers in the chip at 4 ℃ for 20 min. Then cells were permeabilised by incubation for 3 min in 0.2% Triton X-100. After washing with PBS, nonspecific binding sites on the cells were blocked by Block Ace (Dainippon Sumitomo Pharma) for 1 h. The cells were then kept at 4 ℃ overnight in 0.05% Tween 20/PBS containing anti-rabbit ZO-1 antibody (Cat. No. ab59720; Abcam) and anti-mouse RPE65 antibody (Cat. No. MAB5428; Chemicon) at an appropriate dilution. The cells were washed with Tris-buffered saline, followed by immunolabelling with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) and Alexa Fluor 594-conjugated goat anti-mouse IgG for 1 h. F-actin and cell nuclei were stained with rhodamine phalloidin (Life Technologies Corporation) and 4’6-diamidino-2-phenylindole (DAPI; Life Technologies Corporation) for 20 min at room temperature. The images were captured using an image analyser (IN Cell Analyzer 2000) with a 10× objective lens.

References

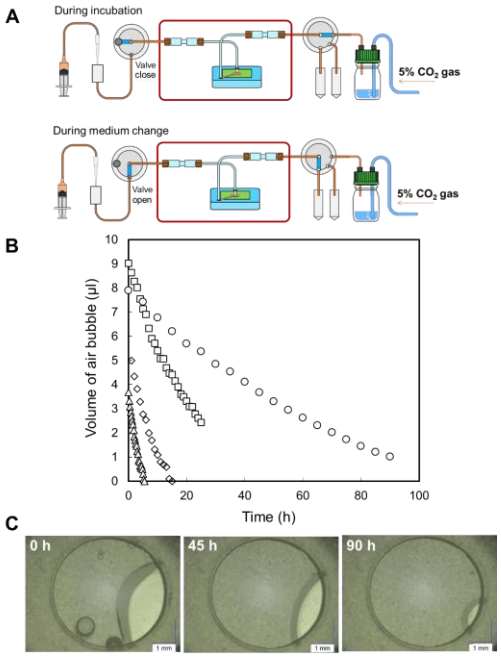
1. Osakada F, Ikeda H, Sasai Y, et al. (2009) Stepwise differentiation of pluripotent stem cells into retinal cells. *Nat Protoc* 4: 811–824.
2. Osakada F, Jin ZB, Hirami Y, et al. (2009) In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. *J Cell Sci* 122: 3169–3179.



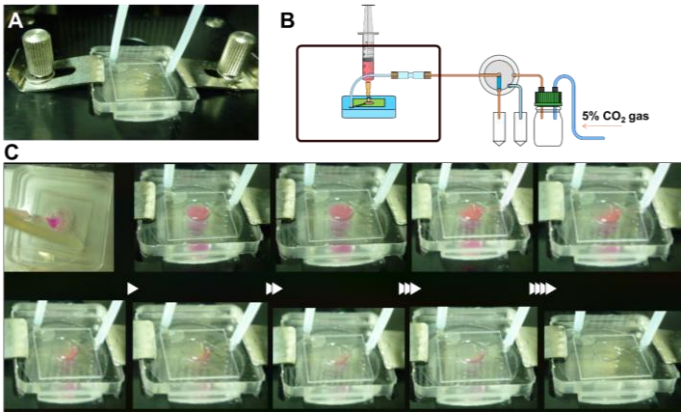
Supplementary Figure 1. Photograph of the aluminum foil sealing machine for storage of media and reagents.



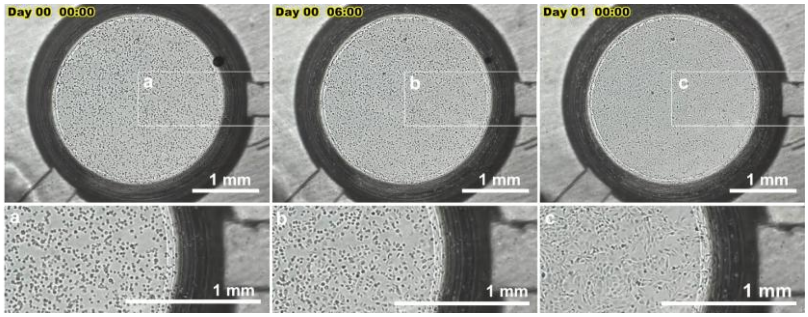
Supplementary Figure 2. Schematic diagram of the control unit of the cell culture system. It consists of three subsystems: an incubation control system, medium supplier control system, and monitoring control system. A custom software routine is used to control important culture parameters including different temperatures, selection of the reservoir for culture medium pumping, and flow rate. Components indicated by arrows are receiving inputs and outward facing arrows represent outputs from that unit.



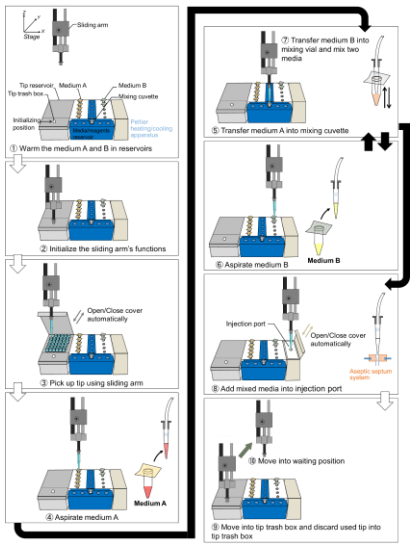
Supplementary Figure 3. (A) Schematic diagram of setup for cell culture chip system with the microfluidic device. Bubbles are artificially created in the source reservoir by pipetting, and water and bubbles were pumped into the collection reservoir. (B) Comparison of evolution of bubble volume over time for bubbles. Symbols; circle, +3 kPa; square, +5 kPa; diamond, +10 kPa; triangle; +20 kPa. (C) Sequential photographs prove that the liquid were successfully removed from the chip at an applied pressure of 3 kPa.



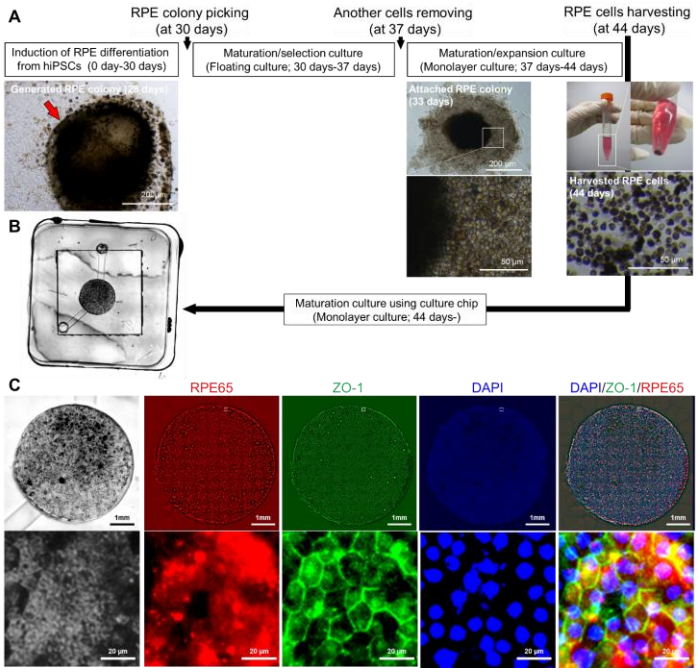
Supplementary Figure 4. Testing the efficiency of pressure-driven medium change in cell culture system. (A) Picture of the cell culture system after assembly. (B) Schematic of a microchip-based device. (C) Photograph showing liquid change process.



Supplementary Figure 5. Homogeneous cell distribution of RPE cells in culture chip system after seeding.



Supplementary Figure 6. Schematic showing how the robotic arm for liquid handling supply culture medium in chip.



Supplementary Figure 7. (A) In vitro differentiation of RPE cells from hiPSCs. (B) Representative images of morphology of hiPSC-derived RPE cells culture in culture chip for 29 days. (C) Immunofluorescence images of tight junction marker (ZO-1, green) and mature RPE marker (RPE65, red). Cell nuclei were stained with DAPI (blue).

Supplementary Video

Video 1. Time-lapse move showing how the sliding arm for liquid handling changes culture medium in an automated chip culture system.

Video 2. Time-lapse movie showing the behaviour and changes in cell morphology of RPE cells during long-term maturation with the automated chip culture system.