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Retina

A Microfluidic Platform for the Time-Resolved Interrogation of Polarized Retinal Pigment Epithelial Cells

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Purpose: Cells grown in milliliter volume devices have difficulty measuring low-abundance secreted factors due to low resulting concentrations. Using microfluidic devices increases concentration; however, the constrained geometry makes phenotypic characterization with transepithelial electrical resistance more difficult and less reliable. Our device resolves this problem.

Methods: We designed and built a novel microfluidic "Puck" assembly using laser-cut pieces from preformed sheets of silicone and commercial off-the-shelf parts. Transwell membranes containing polarized retinal pigment epithelial (RPE) cells were reversibly sealed within the Puck and used to study polarized protein secretion. Protein secretion from the apical and basal surfaces in response to hypoxic conditions was quantified using an immunoassay method. Computational fluid modeling was performed on the chamber design.

Results: Under hypoxic culture conditions (7% O_2), basal vascular endothelial growth factor (VEGF) secretion by polarized RPE cells increased significantly from 1.40 to 1.68 ng/mL over the first 2 hours ($P < 0.0013$) and remained stably elevated through 4 hours. Conversely, VEGF secretion from the apical side remained constant under the same hypoxic conditions.

Conclusions: The Puck can be used to measure spatiotemporal protein secretion by polarized cells into apical and basal microniches in response to environmental conditions. Computational model results support the absence of biologically significant shear stress to the cells caused by the device.

Translational Relevance: The Puck can be used validate the mature phenotypic health of autologous induced pluripotent stem cells (iPSC)-derived RPE cells prior to transplantation.

Introduction

Decoding signaling cascades in living organisms is complicated by the vast complexity and wide range of spatiotemporal scales central to biology. Simplified, physiologically relevant, and scaled in vitro models are needed. Microfluidic platforms have proven useful for constructing hierarchical in vitro model systems, from cell culture to tissues to organs.[1–6] A microfluidic model of the outer retina would be particularly useful, as retinal tissue employs dynamic signaling cascades of exquisite complexity that are poorly understood, given the low concentrations of biomolecules produced and the short time scales of molecular secretions.[7] By sampling locally from polarized retinal pigment epithelial (RPE) cells and maintaining the fluid in small, discrete reaction volumes, it is possible to sample the apical or basal RPE microniche and avoid the sample dispersion and dilution seen with dialysis-based or effluent sampling from bulk "Transwell" studies. Although microfluidic examples of bioreactors for RPE exist, including a device with multiple retinal cell types that adds biological context to the model,[6] they do not permit the normal dynamic flux of molecules secreted from both the polarized apical and basal

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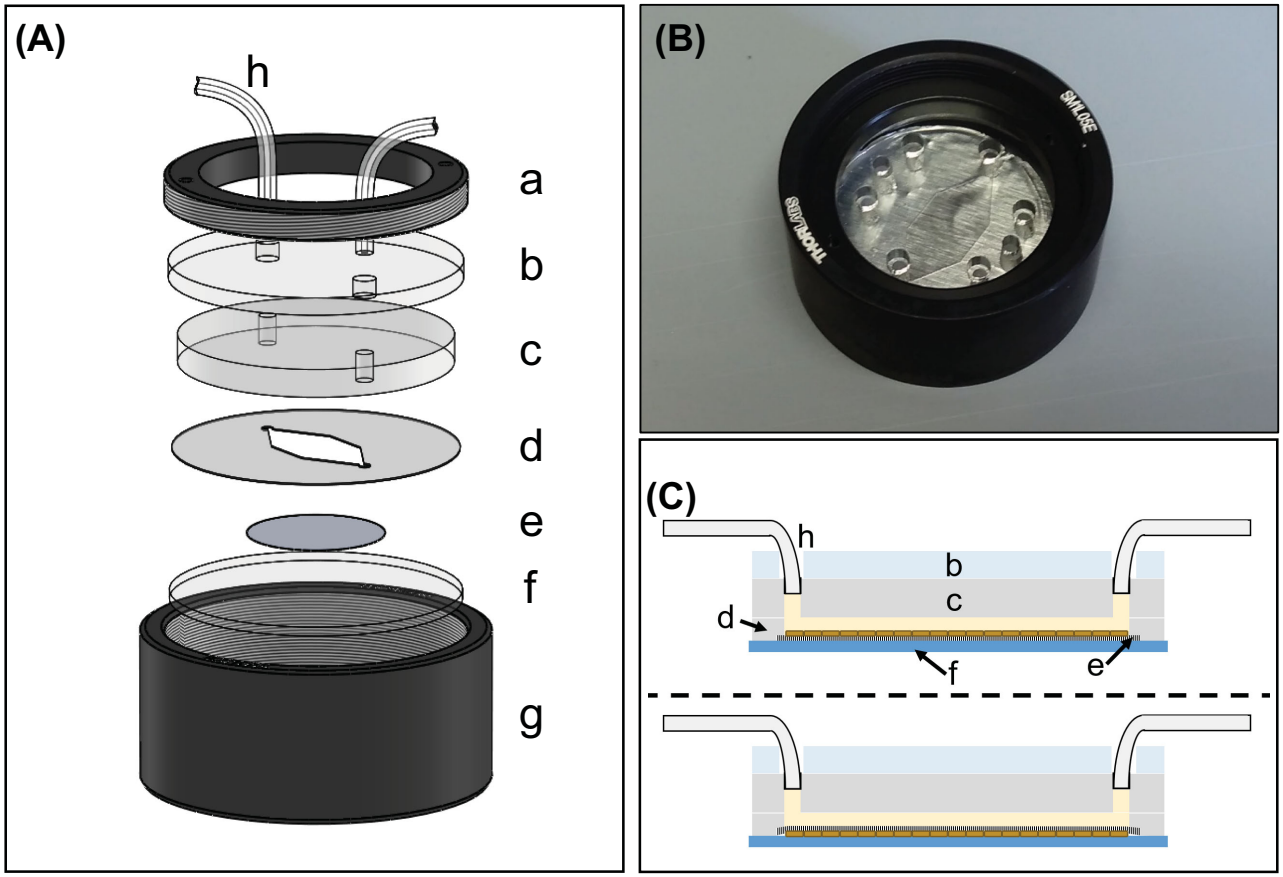

Figure 1. The outer retinal "Puck." (**A**) Exploded diagram of the Puck showing constituent parts. A rotary clamp is made from a 1-in. diameter lens tube (**g**) and retaining ring (**a**). A rigid laser-cut acrylic pressure plate (**b**) translates pressure from the retaining ring to elastic fluidics beneath. The cell chamber lid (**c**) is a laser-cut silicone layer that covers the cell culture region and provides a seal for tubing (**h**) that supplies the device. The cell chamber (**d**) is a laser-cut silicone layer that provides a defined volume of ∼7.6 µL for constant flux cell culture and sits on a cutout from a Transwell membrane (**e**) on which confluent, polarized RPE cells have been grown. The membrane rests on a glass disk (**f**) that provides visualization of the chamber. (**B**) Assembled Puck without cells. (**C**) Schematic diagram of the operation of the Puck for the two modes in which it was used. Letters correspond to the same parts seen in the exploded diagram (**A**). *Top*: polarized RPE cells were grown on a Transwell membrane (**e**) and placed in between the cell chamber cutout (**d**) and the bottom glass disk (**f**). Media flowed into the inlet, over the cells and out the other side. *Bottom*: the Puck was disassembled, and the membrane with polarized RPE cells was inverted to expose the basal side of the cells to the cell chamber. Flow was reestablished and used to measure basal secretions after equilibration in control conditions.

milieus to be determined with high spatiotemporal resolution. Ideally, the local microenvironment that the epithelial cells experience could be modulated experimentally (e.g., by varying oxygen tension or hypoxic stress) and monitored in situ, permitting clear correlations between an experimentally defined environment and cellular dynamic responses.

In this article, we introduce a novel "Puck" microfluidic device (Figs. 1 and 2) that models the outer retina and RPE milieu by means of an easy-to-assemble, microfluidic, continuous flow bioreactor made without the use of soft lithography or indeed without any molding at all. Our approach builds upon previous designs for simulating in vitro tissue models in microfluidic-based devices.[4,8–11] The concept for this work is a readily assembled device that enables rapid and high-sensitivity biochemical characterization of the secretions from a cell-loaded membrane with precultured, phenotypically mature, and polarized adhered cells, cut from a 24-mm Transwell insert and inserted into the Puck. Once this membrane is inserted and reversibly sealed into the Puck, the assembly acts as a continuous-flow microbioreactor that allows withdrawal and sampling of discrete reaction-volume samples of apical or basal secretions from epithelial cells in situ. Key to both the molecular sensitivity and time resolution of samples from the Puck is the small, 7.6-µL volume of the cell chamber. If one considers the difference in volumes between the microfluidic chamber and the volumes used for a standard

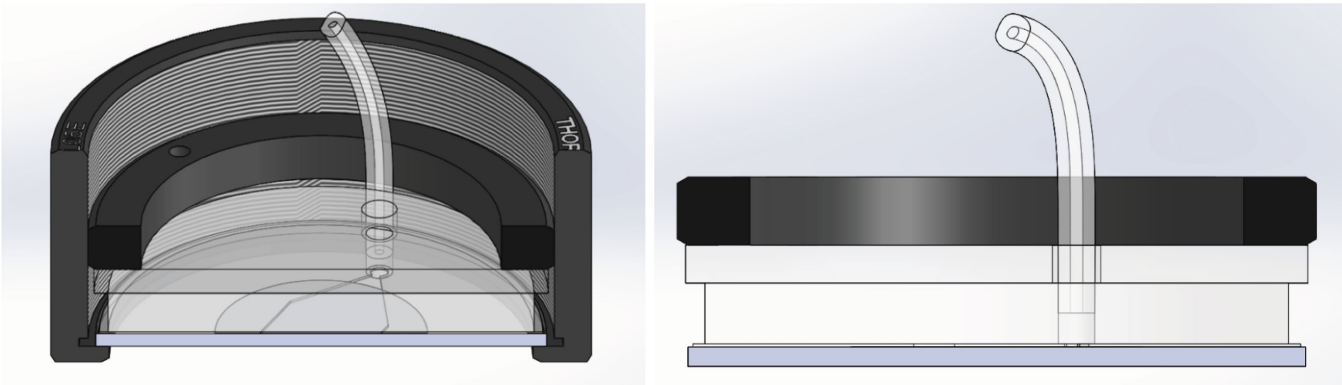

Figure 2. Cross section of Puck assembly showing the layers in Figure 1 to scale (*left*). The semicircular shape at the bottom of the Puck is the Transwell membrane that has been inserted into the device. The trapezoid overlying it is the fluidic chamber. *Right*: side view of the layers of the Puck, without the clamp. The thin layer between the end of the tubing and the glass support (*blue*) is the chemostat comprising the RPE cell monolayer on the membrane and the fluidic chamber (*red arrows*).

24-mm Transwell insert (1.5 mL apical and 2.6 mL basal), the Puck offers a ∼200-fold decrease in volume on the apical and a ∼350-fold decrease in volume on the basal side, respectively. For any given secretion rate and detection threshold for a molecule of interest, these volume ratios translate directly to an improvement in time-resolved detection and dynamics. This platform establishes a real-time biochemical mapping method that enables sampling from polarized, phenotypically defined cells. In this article, we demonstrate the utility of the Puck for near real-time nanomolar-scale detection and measurement of polarized vascular endothelial growth factor (VEGF) secretion under conditions of experimental hypoxia using a commercially available fluorescent assay. We discuss planned future modifications of the Puck design that will permit simultaneous apical and basal analyses, together with in situ transepithelial electrical resistance (TEER) measurements.

Methods

Cell Culture and Polarization

Human donor retina pigment epithelial cells (designated HPE17) were obtained from the Midsouth Eye Bank, Memphis, TN, and cultured as described previously.[12] Dulbecco's modified Eagle's medium (DMEM)/F12 cell culture media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) plus L-glutamine, penicillin, and streptomycin in an atmosphere of humidified 95% air and 5% CO_2 at 37°C. The cells were grown to confluence on 24-mm Transwell permeable support inserts. Once confluent, the cells were maintained in polarization media (using 1.0% fetal bovine serum). The cell culture media was changed every 3 days for up to 6 weeks. Samples of the conditioned media withdrawn from the Transwell at the end of the culture phase were used to prime the tubing and culture the cells in the Puck. To determine functional polarization in the Transwell, TEER between the basal and apical chambers was measured beginning at 2 weeks of culture under confluent conditions. TEER of the polarized RPE monolayer in the Transwell increased over the 5-week observation period to a value of ∼600 $\Omega \cdot cm^2$, indicating good integrity of the cell layer (Supplementary Fig. S1). RPE cell TEER is considered mature when it reaches a consistent level of ∼500 $\Omega \cdot cm^2$. For the relative hypoxia experiments, cells were incubated at 37°C in a controlled environment of 5% CO_2, 7% O_2, and 88% N_2 for the specified time periods.

Sample Collection

The initial Puck design is a single-chamber device wherein only one side of the membrane is perfused at any given time. To examine secreted proteins from both the apical and basal sides of the membrane, a sequential culturing method was performed. With the apical side facing up, the membrane disk was inserted into the device such that the basal side faced down and toward the glass disk that had been preplaced in the clamp (Figs. 1A, 1B). Next, the chamber layer was inserted, followed by the silicone chamber seal and an acrylic pressure plate. The Puck was then compressed with the retaining ring. Tubing was inserted into the two ports, with one tube connected to a pump that provided inflow to the chamber and the other to a tube that directed the chamber effluent into a media reservoir. The assembled Puck and an open media reservoir were then placed inside an EVOS FL Auto

microscope stage-mounted incubator (Life Technologies, Carlsbad, CA). The cells were cultured under 5% CO_2 at 37°C for 4 hours at ambient oxygen saturation. For the VEGF studies, the oxygen level was reduced from 20% to 7% and equilibrated for 30 minutes to create a hypoxic environment in the chamber. The fluid flow was 5 µL/min. For each hour run, the pump was turned off for 30 minutes, then run for the other 30 minutes to collect conditioned-media samples on ice. The apical side was sequentially sampled for 4 hours (Fig. 1C, top). The Puck was then disassembled and reassembled with the Transwell membrane inverted, basal side up, and the cell chamber was reinstalled, again chamber down (Fig. 1C, bottom). The pump was reconnected to the media reservoir and the study performed in the same manner as for the apical side following 4 hours under basal O_2 conditions. At each time point, a total of ∼150 µL of sample was collected. All samples were stored at −80°C for the immunoassays. A higher-resolution cross section of the Puck is shown in Figure 2.

Immunoassay Methods

We used a no-wash, bead-based chemiluminescence immunodetection method, AlphaLISA, to detect and quantitate target molecule secretion from each side of the epithelial membrane in the device. AlphaLISA uses a fluorescent proximity assay in which binding of target molecules to antibodies captured on 250- to 350-nm beads leads to an energy transfer from a donor bead to the acceptor, producing a luminescent signal. The luminescent signal is directly proportional to the concentration of the analyte in solution and is generated by laser excitation at 680 nm. A human VEGF kit (PerkinElmer, AL201C, Waltham, MA) was used. The standard dilutions were performed according to the manufacturer's protocol. AlphaLISA signals were detected using an EnVision multimode plate reader (PerkinElmer) at 615 nm detection. Then, 10 µL of 5× Anti-Analyte Acceptor beads at a 10-µg/mL concentration was added and incubated for 30 minutes at room temperature. Next, 10 µL of 5× biotinylated antibody antianalyte was added (1.0 nM final concentration) and incubated for 60 minutes at room temperature, and 25 µL of 2× SA-Donor beads at a 40-µg/mL concentration was added and incubated for 30 minutes at room temperature in the dark. A four-parameter logistic regression curve was fitted to standard dilution data, as recommended in the manufacturer's protocol, using MATLAB (The MathWorks, Natick, MA, USA) via a custom script (see Supplemental Information). As tested, the assay had a lower limit of detection between 1 and 3 pg/mL (Supplementary Fig. S2). We determined the sensitivity and dynamic range for the assay by serial dilution of protein standards in 5-µL examination volumes. We quantified the protein-specific signal from very small volumes of collected, "time-stamped" trains of droplets.

Puck Device Design Fabrication and Testing

The design of the assembled microfluidic Puck permits the use of phenotypically characterized and validated confluent and membrane-adherent cells. For these studies, we used human RPE cells, a highly polarized, hexagonally packed, and pigmented tissue layer beneath the retina that plays a critical role in the maintenance of retinal health and function.[13,14] The RPE cells were cultured on Transwell inserts (Corning Costar Transwell 3412; Corning, NY) and maintained in a polarized and pigmented epithelial morphology (as seen in the native tissue) for 30 days, while TEER was monitored (EVOM2; World Precision Instruments, Sarasota, FL). The tissue polarization was quantified using TEER measurements[15–17] prior to cutting the Transwell membrane and the cells it supports from the Transwell insert by means of a biopsy punch (Acu. Punch CE0413; Acuderm, Inc., Ft. Lauderdale, FL). The cell-laden membrane was transferred en bloc to the microfluidic device for experiments. The transfer and study of the cell-laden Transwell membrane facilitated detailed examination of an intact monolayer phenotype with mature tight junctions. The microfluidic chamber was designed to allow the disk-like Puck components to be stacked within a threaded tube and a single retaining ring clamped together as a multilayer assembly, to incorporate the membrane with polarized cells into the restricted-volume microfluidic chamber. The Puck microbioreactor was designed to achieve the same function as commercially available openable flow-through microfluidic devices.[18,19] Rather than purchasing clamps made from machined parts that use proprietary designs, we used commercially available lens tubes (SM1L05E; ThorLabs, Newton, NJ) and threaded retaining rings (SM1PRR; ThorLabs) as the basis of a cylindrical clamp. Multiple layers of microfluidic elements were laser-cut from preformed acrylic (8560K172; McMaster-Carr, Elmhurst, IL) and silicone (87315K71 and 87315K73; McMaster-Carr) sheets using an infrared laser cutter (Mini 30W; Epilog Laser, Golden, CO). Unlike other low-volume, membrane insert devices in the literature, our approach precluded the need for either molding processes, such as those used to make soft lithography with PDMS[20] or thermoplastics,[21] or the need for three-dimensional printing.[22] A Transwell membrane supporting a polarized monolayer of RPE cells was cut out of a Transwell

insert with a biopsy punch to create a 12-mm diameter membrane disk covered with cells. The layers b, c, and d shown in Figure 1 were inserted into the lens tube, and these silicone layers were used to compress a chamber over the membrane, backed by a glass disk (201080; Esslinger & Co., St. Paul, MN), using a spanner wrench (SPW801; ThorLabs), and tightened to a torque of 0.11 to 0.22 N-m. DMEM media with 10% fetal calf serum were drawn into a patented, custom-built rotary peristaltic pump[23] from a sterile reservoir and connected to the Puck using 1.5-mm Tefzel tubing (1/16-in. OD, 0.02-in. ID, 1516L; IDEX Health & Science, West Henrietta, NY). The cells outside the margins of the reservoir remained sandwiched between silicone and silicone-backed membrane. To minimize any impact of the contents of damaged cells leaking into the test chamber, the chamber was perfused with media after insertion of the membrane, prior to collection of the test samples, to rinse away potential contaminants. A patented custom bubble trap was placed between the pump and the Puck to prevent evolved gas bubbles from entering the cell chamber.[24] Computer-aided design (CAD) files for the laser-cut parts can be found at https://github.com/ericspivey/retina-puck.

Computational Fluid Modeling

The CAD file used for fabrication of the cell chamber was imported into the finite element modeling software COMSOL, which was used to model both diffusion in a no-flow state and flow through the device at 5 µL/min. The height of the chamber was based on the 125-µm thickness of the silicone sheet used to fabricate the chamber. Water was selected as the material contained by the channels and chambers within the COMSOL model. The diffusion rates were calculated using the "transport of dilute species" physics module, while the "creeping flow" physics module (a laminar flow module that neglects Stokes flow) was used to compute the flow. Model files can be found at https://github.com/ericspivey/retina-puck for a more detailed understanding of the modeling, and key model parameters are included in Supplementary Table S1.

Statistical Analysis

VEGF standard curve and each time point levels were statistically analyzed with linear regression. One-way analysis of variance, Student's *t*-test was performed with VEGF concentrations and reported as mean ± standard deviation. P values of ≤ 0.05 were considered statistically significant.

Results

Polarized VEGF Secretion

Under hypoxic culture conditions, basal VEGF secretion by polarized RPE cells increased significantly from 1.40 to 1.68 ng/mL at 2 hours ($P < 0.0013$) and remained stably elevated through the 4-hour time point (Fig. 3). Conversely, VEGF secretion from the apical side remained relatively constant under the same hypoxic conditions, from 1.47 to 1.52 ng/mL.

Puck Flow Modeling

Finite element modeling rendered in COMSOL showed that the Puck provided a 25-fold increase in the modeled secreted protein concentration over the modeled concentration expected in the apical chamber of a Transwell (Supplementary Fig. S3). Results of finite element modeling under laminar flow conditions (Fig. 4) show that pressure and velocity were uniform throughout the central area of the flow chamber in which the polarized RPE cells were placed (Figs. 4A, 4B) and that laminar flow was maintained (Fig. 4C). Chamber volume was calculated to be 7.6 µL. Assuming a viscosity of 0.94 cP for the media,[25] shear stress at the chamber surface was ~5.0 mPa. If we assume that cells reduce the thickness of the chamber by up to 10 µm, the shear stress at the surface of a chamber 115 µm thick was ~5.9 mPa (Supplementary

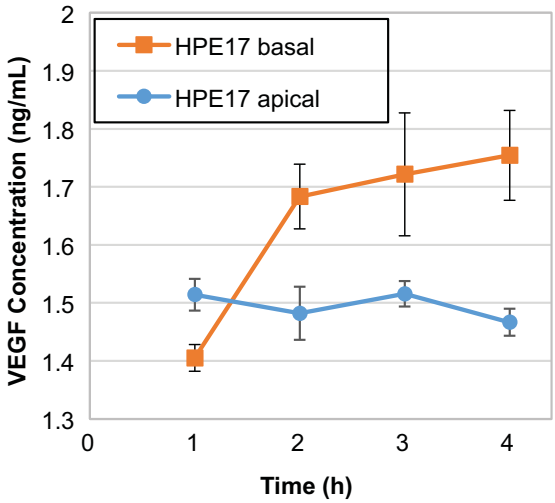

Figure 3. VEGF secretion from polarized RPE cells (HPE17) over 4 hours, under hypoxic conditions (7% O_2). Time was started at the initiation of hypoxic culture conditions; basal secretion rises significantly ($P < 0.0013$) and exceeds apical secretion at 2 hours. Measurements of apical (*blue*) and basal (*orange*) secretion were calibrated using four-parameter logistic regression calculated from a series of concentration standards. *Error bars* show standard deviation ($n = 3$).

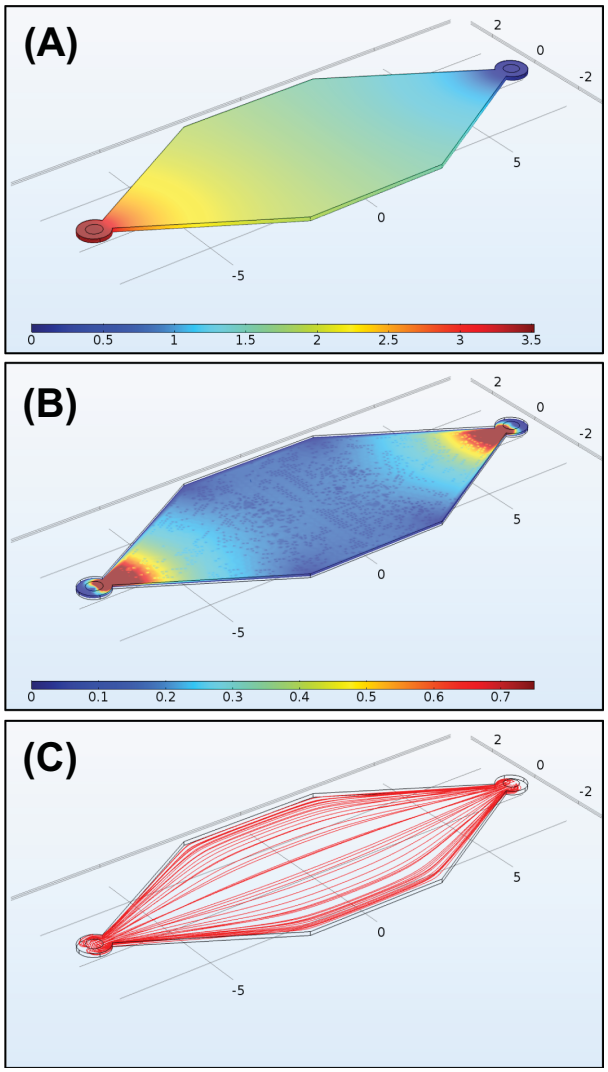

Figure 4. Flow modeling of the Puck fluidic chamber (axes in millimeters). (**A**) Pressure driving the flow of media through the Puck with a flow rate of 5 µL/min; units on the legend are Pascals. (**B**) Linear velocity of media through the chemostat, measured at the center plane. Units on the legend are mm/s. (**C**) Streamlines through the Puck illustrate the laminar nature of flow in the cell chamber.

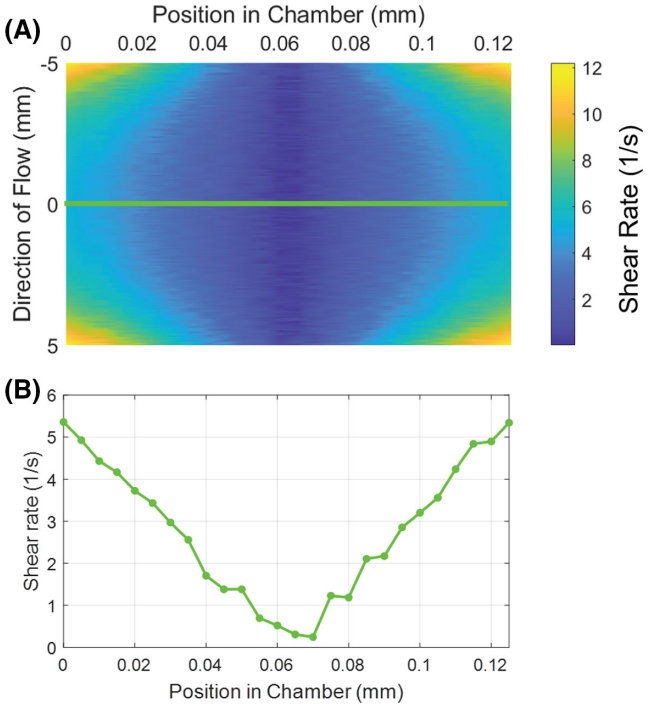

Figure 5. Calculated shear stress exposure. (**A**) Surface plot of shear rate exported from COMSOL computational model, taken along the central 1 cm of the midline of a 125-µm chamber, where cells are located during the flow experiments. Axes are not to scale and represent the cell chamber, with height within the chamber on the x-axis ("0" at the bottom surface of the chamber) and flow direction on the y-axis (media flows in along the vector from "−5" to "5"). Flow rate is 5 µL/min. *Green line* is at "0" and illustrates the path of the shear rate plot shown in B. (**B**) Plot showing shear rate as a function of height within the chamber at the center of the cell chamber.

Fig. S4). Therefore, the range of shear stress expected to be experienced by most cells at a flow rate of 5 µL/min was between 5.0 and 5.9 mPa (Fig. 5).

Discussion

To date, studies on the polarized secretion of proteins by the RPE have been limited by the available methods, which detect spatially and temporally averaged responses in bulk fluid using Transwell tissue culture plates. However, mature polarized RPE cells can be grown in smaller volume chambers.[26] An early true microfluidic device for the coculture of RPE and endothelial cells examined VEGF expression in response to low glucose and hypoxia, but the RPE cells could not be fully differentiated in the device, despite long-term culturing.[27] The design of the Puck microbioreactor permits the use of well-characterized RPE cells (and other epithelial cell monolayers) for experimental manipulation. This was confirmed here by the polarized, basal secretion of VEGF under conditions of experimental hypoxia.[28] Establishing a microfluidic model for the epithelial tissue microniche, for example, will permit us to examine signaling dynamics in the outer retina, such as responses to chronic versus pulsatile oxidative stress (as seen with smoking), polarized spatiotemporal expression of proangiogenic proteins in the apical and basal microniches, and the time course and dynamics of their secretion. These mechanisms are currently poorly understood in many epithelial tissues, and the improved temporal resolution provided by the Puck may have a significant impact on the visualization of cellular responses to experimental manipulation and, in turn, afford a deeper understanding of the pathophysiology of many diseases.

This platform and method establish a real-time biochemical mapping methodology that permits polarized sampling with high spatiotemporal resolution from small ensembles of phenotypically defined cells reflecting the in situ tissue microniche for experimental manipulation. More broadly, an openable microfluidic device made without the use of photolithography or soft lithography, and that uses commercially available components, is a simple platform with which to study other tissue and cell systems. Although the benefits of using the Puck are considerable, care must also be taken to avoid excessive elution of signaling molecules that are used by cells to maintain their phenotype and function. This can be prevented, however, by considering the time-averaged concentration of relevant signaling molecules when determining the timing of flow and no-flow states during an experiment. Similarly, the introduction of nonphysiologic conditions, such as shear stress in the subretinal space, may induce changes in gene expression through other mechanisms in cells of interest. The shear tolerance of polarized RPE cells has not been studied, but the tight junctions would be expected to offer resistance to such shear forces. Much higher shear stresses (7 or 15 dyn/cm^2 for periods up to 10 hours) are well tolerated by respiratory epithelial cells, and shear stress (4–8 dyn/cm^2) causes few changes in corneal epithelial cell gene expression.[29,30] We therefore suggest that the much lower peak shear experienced by RPE cells in the retina Puck, less than 6 mPa (0.06 dyn/cm^2), is not high enough to significantly affect their function. Although shear forces and stress on RPE cells are probably not relevant in the subretinal space (at least as far as we know), we modeled these forces here to begin quantifying them in the Puck to better understand the potential impact of (modifiable) fluid flow in the device to induce signaling (e.g., through mechanotransduction mechanisms). Future work examining the impact of shear stress on RPE tight junction structure and function in the Puck (e.g., ZO-1, desmosomal cadherins, and the intermediate filament cytoskeletal network in response to fluid flow stress) is planned.

The ability to perfuse native, cultured, or engineered tissue in a low-volume system, where tens of separate experiments could reasonably be run in parallel, offers a potentially powerful tool to researchers in many fields. Future work with the Puck will introduce a two-chamber device permitting flow on both the apical and basal sides of the RPE for simultaneous interrogation of the respective microniches. For this device, a second cell chamber will be added to the basal side of the membrane. To stabilize the membrane between the apical and basal chambers, a cell-compatible adhesive layer will be added to the cell chamber surfaces that face the membrane. This assembly will then be placed between the chamber lid and the glass disk. The lid and top cell chamber will have additional holes to allow perfusion of the bottom cell chamber. The Puck platform can also be integrated with TEER electrodes, which would permit time-resolved analysis of membrane integrity for many cell systems. It has been shown that TEER measurements can vary widely between measurements in Transwells and microfluidics, even using the same cell type.[31] Because chamber geometry and electrode placement greatly influence TEER measurements in microfluidics, our design will seek to minimize any changes to the geometry of the cell chambers while incorporating static sputter-coated electrodes that are integral to the device. A two-chamber Puck with integrated TEER electrodes could be used to validate the comparability of the TEER measurements between current microfluidic culture methods.

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