A PHOTOGRAMMETRIC METHOD TO MEASURE FLUID MOVEMENT ACROSS ISOLATED FROG RETINAL PIGMENT EPITHELIUM

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ABSTRACT Bullfrog retinal pigment epithelium (RPE)/choroid explants were mounted in an Ussing chamber modified so that the side bathing the choroid was completely sealed. Net fluid movement across the RPE and into or out of the sealed side of the chamber forced the tissue to assume a convex or concave shape. Photogrammetry was used to measure this tissue bowing by analyzing the displacement of the image produced on the RPE surface by an off-axis laser. Under baseline conditions, measured net fluid movements were always in the retina-to-choroid direction with a mean of 7.6 $\mu l\ cm^{-2}\ h^{-1}$. Potential artifacts from a variety of sources have been examined and shown to be insignificant.

INTRODUCTION

The retinal pigment epithelium (RPE) is a single layer of cuboidal epithelial cells that lie subjacent to the retina (1). The transepithelial transport properties of RPE from a number of species have been studied (2–5). Two laboratories have described techniques to measure volume changes on both sides of bullfrog RPE/choroid preparations mounted in Ussing chambers[1] (6). They reported spontaneous net *trans*-RPE fluid fluxes in the retina-to-choroid direction of 4.8 $\mu l\ cm^{-2}\ h^{-1}$ (7) and 7.3 $\mu l\ cm^{-2}\ h^{-1}$ (8).

The general technique employed by these groups has previously been used to measure transepithelial fluid movement across other epithelia such as frog choroid plexus (9) and dog tracheal epithelium (10). The essential feature of these techniques consisted of sealing both sides of the Ussing chamber except for small, vertical columns of fluid that extended from either side. The net transepithelial volume change was calculated from the changing heights of these fluid columns. Unfortunately, as the heights of these fluid columns changed, the hydrostatic gradient across the tissue in the Ussing chamber also changed. This pressure change could force the tissue to bow forward or backward within the chamber and this movement would reduce the calculated value of transepithelial volume change.

In the case of bullfrog RPE, where the reported rate of fluid movement (4.8–7.3 $\mu l\ cm^{-2}\ h^{-1}$) is equivalent to a volume of fluid only 48–73 μm thick traversing the RPE per hour, very small tissue movements can substantially alter the measured rate of net *trans*-RPE fluid flux. Another problem with the previously used technique is that changes in the physical properties of the RPE or choroid may produce movement of the tissue within the chamber that would change the heights of the fluid columns and be interpreted by the measuring device as net *trans*-RPE fluid movement. Such an artifact has recently been reported (11).

We have developed a new method to measure net *trans*-RPE fluid flux. An Ussing chamber was modified so that tissue movements within the chamber reflect only *trans*-RPE volume change. Quantification and calibration of the rate of tissue movement within the Ussing chamber yields a measure of the rate of *trans*-RPE fluid flux free of the potential artifacts described above.

MATERIALS AND METHODS

Bullfrog (*Rana catesbeiana*) RPE/choroid explants were prepared as described elsewhere (4, 5). The choroid is an ocular tissue located between the RPE and the sclera. It is a network of fine blood vessels in a loose stroma that intermeshes with the RPE basement membrane. Bullfrog RPE/choroid explants are thin and very pliable.

The tissue explants were mounted in an Ussing chamber modified so that the side bathing the choroid was completely sealed. Four ports constructed of Teflon tubing with gaskets of silicone rubber were placed in the sealed portion of the chamber. Two ports accommodated conventional Ussing chamber electronics (a PtIr wire and an Ag-AgCl electrode connected via an agar bridge). The third port was connected to a glass syringe driven by a hydraulic microdrive (model 607 W; David Kopf Instruments, Tujunga, CA) so that small volumes of fluid could be injected or withdrawn. The fourth port was opened to the other side of the chamber when we wanted to set the hydrostatic gradient across the tissue to zero.

The volume of fluid in the sealed half of the chamber and in its connecting tubing was ~1 ml. The sealed portion of the chamber was constructed of Kel-F plastic, a homopolymer of chlorotrifluoroethylene (3M Corporation, St. Paul, MN) and immersed in a covered, glass-lined,

[1]An Ussing chamber is a device in which epithelia are mounted between two electrically isolated saline solutions so that the transepithelial voltage and the tissue's electrical resistance can be measured.

4-liter water bath that formed the other side of the chamber (Fig. 1). The area of exposed tissue was 0.07 cm^2.

The bathing medium consisted of (in millimoles per liter): 110 Na, 2.0 K, 1.8 Ca, 1.0 Mg, 90.1 Cl, 27.5 HCO_3, and 10 glucose. A water-saturated 95:5% O_2/CO_2 gas mixture was blown over the top of the 4-liter water bath to maintain a pH of 7.4 ± 0.1.

The chamber, maintained at room temperature, was insulated with air gaps and one-quarter inch Plexiglas, which limited temperature changes during an experiment to <0.1°C h^{-1} in spite of room temperature fluctuations of as much as several degrees in an hour. The bathing medium used in each experiment was added to the chamber and equilibration with 95:5% O_2/CO_2 atmosphere was begun 10–16 h before the RPE/choroid explant was obtained. The chamber design permitted us to seal the RPE/choroid explants within it without touching the apparatus with our hands. These precautions seemed to eliminate thermal gradients within the apparatus.

The transepithelial electrical potential (V_e) generated by the RPE was measured with a pair of Ag-AgCl electrodes connected to either side of the preparation via agar bridges and to a direct current (DC) amplifier (model 16B; Grass Instrument Co., Quincy, MA). Current-passing electrodes consisted of bare platinum-iridium wires immersed in the bathing medium. Current pulses of 15–25 $\mu A\ cm^{-2}$ and 2–5 s duration were developed by a stimulus isolater (Word Precision Instruments, Inc., New Haven, CT) and passed through the RPE-choroid at 5–10 min intervals. The output from the stimulus isolator and the V_e were continuously recorded on a dual channel chart recorder (model 220; Gould Inc., Recording Systems Div., Cleveland, OH). The electrical resistance of the preparation was calculated from the change in V_e induced by each current pulse. Under these conditions, the RPE/choroid behaved as an ohmic resistor.

Tissue bowing was measured by projecting a 0.5 mW HeNe laser beam (model 155; Spectra-Physics Inc., Mountain View, CA) onto the retinal side of the RPE/choroid at an angle of ~60° from normal. Before the laser beam struck the RPE, it was partially interrupted by a portion of the chamber, thus casting a shadow and creating a sharp light/dark interface on the RPE surface. This interface was viewed with a 50× dissecting microscope linked to a TV camera (model WV-1350A; Panasonic Co., Secaucus, NJ) through a 2× adapater. The microscope was oriented so that its optical axis was ~30° from normal to the RPE surface and 90° to that of the incident laser beam. The position of the interface shifted as transepithelial fluid movement forced the tissue to bow forward (Fig. 2). A shutter blocked the laser beam at all times except when measurements were actually taken.

In initial experiments, the optics of the apparatus were oriented so that the image of the light/dark interface was horizontal on the TV monitor. At time zero, the output of the TV camera was entered into channel one of a two-channel signal averager (model 4202; EG & G Princeton Applied Research, Wellesley, MA). Typically 200 TV frames were entered into the averager over 7 s. At subsequent 3-min intervals, the TV signal was entered into channel two and the averaged signal was subtracted from that stored in channel one. As the light/dark interface moved, a square wave, with its width dependent upon the distance the interface moved, appeared in the output from the signal averager. This square wave was displayed on an *X-Y* plotter (model 7015B; Hewlett-Packard Co., Palo Alto, CA) both in unaltered form and after being passed through an electronic integrator. The change in the integrated output of the signal averager was used as the index of net transepithelial fluid movement. More recently, we have coupled the video camera to a microcomputer (IBM-PC; IBM, Inc., Boca Raton, FL) via a digitizing interface ("Video Van Gogh", Tecmar Corp., Cincinnati, OH) to more precisely locate the position of the light/dark interface at 2 min intervals. A one-dimensional intensity profile was obtained by averaging the digitized image perpendicular to the light/dark interface. The position of the light/dark interface was assigned to the point on the one-dimensional intensity profile with the greatest slope.

Calibration was performed after each determination of the rate of volume change by withdrawing measured volumes of fluid from the sealed half of the chamber with a glass syringe driven by a hydraulic microdrive. At least three (typically five to six) determinations of tissue position vs. volume withdrawn were used in each calibration. It was essential to calibrate the system as part of each experiment because the precise geometry of the laser beam, microscope, and tissue varied from experiment to experiment. The volume of fluid equivalent to a certain amount of movement of the light/dark interface could vary as much as 50% when the geometry of the apparatus was changed.

RESULTS

To test the stability of the apparatus, a piece of thin latex rubber (dyed black to have the same reflectance as bullfrog

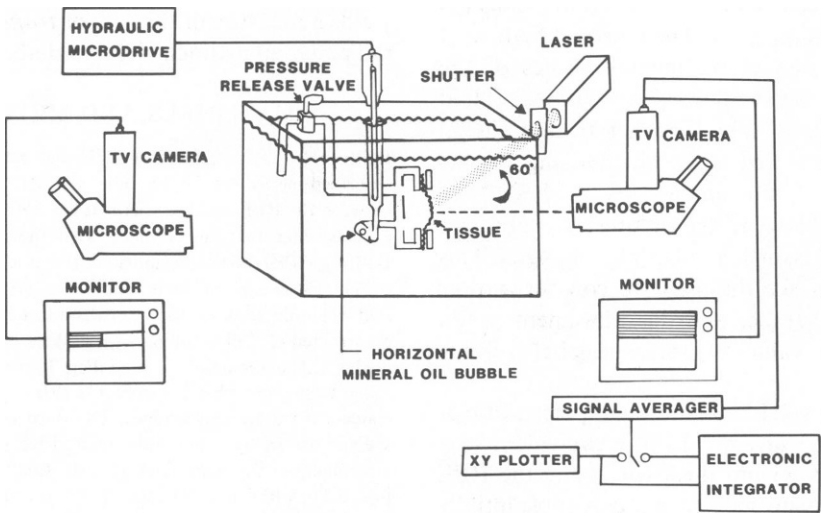

FIGURE 1 Schematic of the apparatus. The sealed portion of the chamber (volume = 1 ml) sits within a 4-liter water bath that also forms the other side of the chamber. The laser, microscope, TV camera, monitor, and electronics on the right were used to measured tissue movement. The syringe, hydraulic microdrive, microscope, TV camera, and monitor on the left were used to correlate tissue movement with volume changes. Not shown are conventional Ussing chamber electronics and the water-saturated 95:5% O_o/CO_2 gas mixture blown over the top of the apparatus.

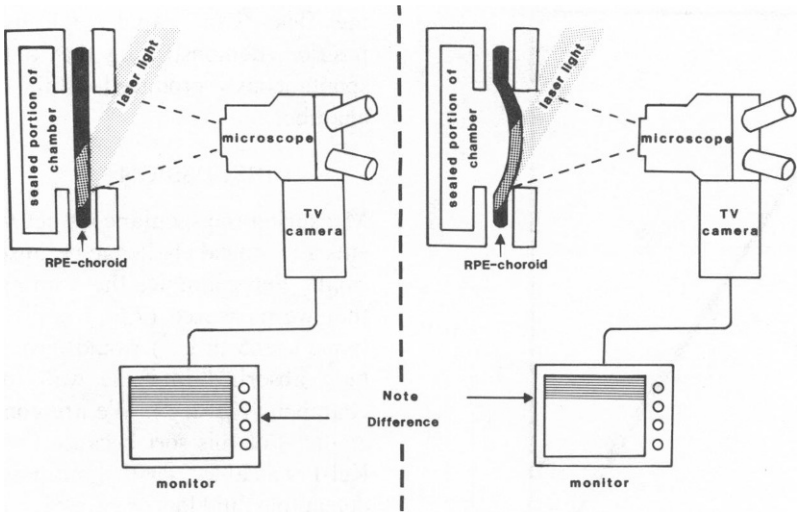

FIGURE 2 Photogrammetric principle. As fluid entered the sealed portion of the chamber, the tissue assumed a convex shape (*right* part of the figure). This bowing (greatly exaggerated for purposes of illustration) was measured by analyzing the displacement of the image produced on the RPE surface by an off-axis laser.

RPE) was sealed in the chamber in place of the RPE/choroid explant. Under these conditions, the sealed portion of the chamber withstood pressure gradients estimated to be as great as 2 atm without a detectable fluid loss or gain over a period of 48 h. Under high pressure, the rubber became translucent and expanded far beyond the limits of the movement detection system so that changes in volume were detected by inspection only. At substantially lower pressures, the very sensitive movement detection portion of our apparatus was used. At these low pressures, injecting or withdrawing small fluid volumes (~0.1 μl) moved the rubber membrane to a new stable position. Opening the pressure relief valve caused the membrane to fall backwards, thus verifying that the chamber was pressurized.

In preliminary experiments with a prototype chamber constructed of acrylic plastic rather than Kel-F, there was always a spontaneous fluid movement, apparently into the chamber walls. The rate of fluid absorption was 1 $\mu l\ h^{-1}$, even after the acrylic chamber had equilibrated in experimental solution for 30 d. Kel-F seems to be the only plastic that possesses the required dimensional stability for these experiments and does not absorb water (12). Teflon tubing was used because its walls are relatively rigid and absorb practically no water (13).

During calibration, the position change of the light/dark interface on the TV screen was linearly related to the volume of fluid injected or withdrawn from the sealed portion of the chamber. With latex rubber in the chamber, the slope of repeat calibration curves always varied by <5%. Similar results were obtained with the RPE/choroid explants in the chamber. Fig. 3 shows four calibration curves obtained over a 5-h period for the system containing an RPE/choroid explant. During this period, the geometry of the tissue, laser, and microscope was not changed. The slopes of these four curves varied by 2.7%.

When the volume of fluid that spontaneously traversed the RPE/choroid was measured repeatedly, the rate of volume change was constant for 60 min (Fig. 4). In some experiments, the rate of measured volume change with time began to slow after an hour. The experiment illustrated at the bottom of Fig. 5 shows this effect. If the pressure relief valve was temporarily opened (thus resetting the hydrostatic gradient across the tissue to zero), or the tissue brought back to its original position by calibrating (also resetting the hydrostatic gradient to zero), the rate of measured fluid movement returned to its initial value. For this reason, measurements of fluid movement were usually made for an hour, the system calibrated, the pressure relief valve opened briefly (to verify that the initial hydrostatic gradient across the tissue was zero), and another set of fluid movement measurements made. We found that the rate of fluid movement correlated with the electrical activity of the RPE. Therefore, we made repeated sets of measurements for as long as the RPE electrical activity remained constant. Typically this was two or three sets of measurements per RPE/choroid explant but six repeated sets of measurements were made on one explant on one occasion. For 20 such experiments, the net *trans*-RPE fluid flux was always in the retina-to-choroid direction with an average rate of 7.6 (SD = 4.3) $\mu l\ cm^{-2}\ h^{-1}$, the average spontaneous transepithelial voltage was 9.4 mV (retina side positive), and the average transepithelial resistance was 212 $\Omega\ cm^{-2}$.

When an RPE/choroid explant was sealed in the chamber and the pressure relief valve left open, there was essentially no spontaneous tissue movement. However, if the pressure relief valve was then closed, the tissue abruptly began to move ($n = 6$). Fig. 5 shows three of these experiments. One of these experiments was terminated by reopening the pressure relief valve rather than by calibrat-

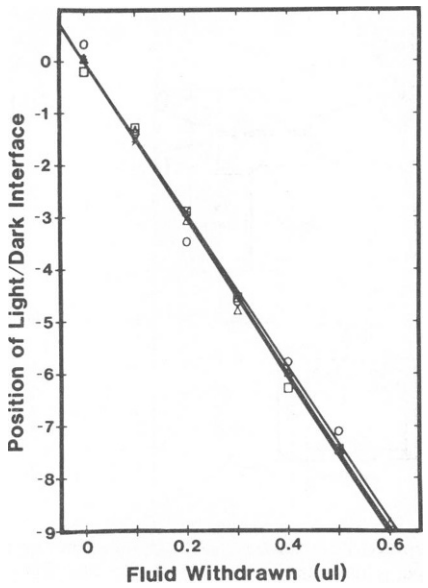

FIGURE 3 Calibration curves. Volumes of fluid withdrawn from the sealed side of the chamber vs. the position of the light/dark interface on the TV screen for four different calibrations of the same RPE-choroid preparation made over a 5-h period. The geometry of the tissue, laser, and microscope was identical for these four calibrations. Symbols * for time = 0; ○ for time = 1 h; △ for time = 3 h; and □ for time = 5 h.

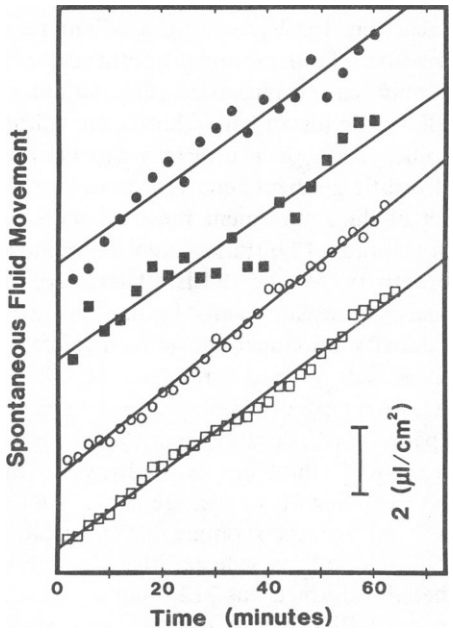

FIGURE 4 Experimental curves. Volume change per centimeter squared of exposed RPE surface in the sealed half of the chamber spontaneously produced by four different RPE-choroid explants over time. The top two curves were obtained using the older image-analysis technique employing the signal averager. The bottom two curves were obtained using the microcomputer. For clarity, these four curves are separated vertically. The vertical scale is indicated in the *lower right* corner of the figure.

ing. The tissue rapidly fell back towards its original position, demonstrating that a hydrostatic gradient was spontaneously produced within the sealed portion of the chamber.

DISCUSSION

Measuring the spontaneous net *trans*-RPE fluid flux presents a technical challenge because the volume change is so small. For example, the transepithelial fluid movement that we measured (7.6 μl cm^{-2} h^{-1} × 0.07 cm^2 exposed tissue = 0.5 μl h^{-1}) would have been totally obscured by fluid absorbed into the walls of our prototype acrylic chamber (1 μl h^{-1}). We are confident that we abolished artifacts of this sort because the chamber constructed of Kel-F was able to withstand pressure gradients without a detectable fluid loss or gain over a period of 48 h. Artifacts due to thermal gradients, dimensional instability of the materials used in the apparatus, leaks, etc., would also have been apparent during these experiments.

It has recently been reported that measurements of RPE-mediated fluid movement using RPE/choroid explants and the type of chamber previously described in the literature can contain large artifacts due to changes in the thickness of the choroid independent of RPE-transport (11). This is not a problem for measurements made with the chamber described in this report because the choroid is totally enclosed within the sealed portion of the chamber. While the choroid may become edematous or its blood vessels may contract or dilate, the fluid gained (or lost) by the choroid will come from (or go into) the nonchoroidal space of the sealed portion of the chamber. Thus, the total volume of the sealed portion of the chamber will not change and the tissue will not move within the chamber.

It is possible, however, that a physical change in the choroid could produce a hydrostatic gradient across the RPE (e.g., by pulling the RPE backwards from a slightly bowed shape or by pushing the RPE forward as the cells in the choroid become edematous). This hydrostatic gradient would drive a net fluid flux across the RPE at a rate proportional to the product of the hydrostatic gradient and the conductance of the system to bulk fluid flow between the two chambers. The experiment illustrated in Fig. 5 demonstrates that there was no artifact of this sort when the tissue was bathed in physiologic medium. When the pressure relief valve was open, the conductance of the system was very large and this artifact would be maximum. Yet under these conditions, there was essentially no tissue movement. When the valve was closed and the conductance of the system was very low, the tissue immediately bowed forward and a hydrostatic gradient was created. This indicates that movement of the tissue in the chamber was in response to a hydrostatic gradient rather than the other way around.

It is possible that the RPE cells themselves could swell, forcing the RPE apical surface to move forward. Note that the RPE is a monolayer of cells <10 μm thick (1) so these

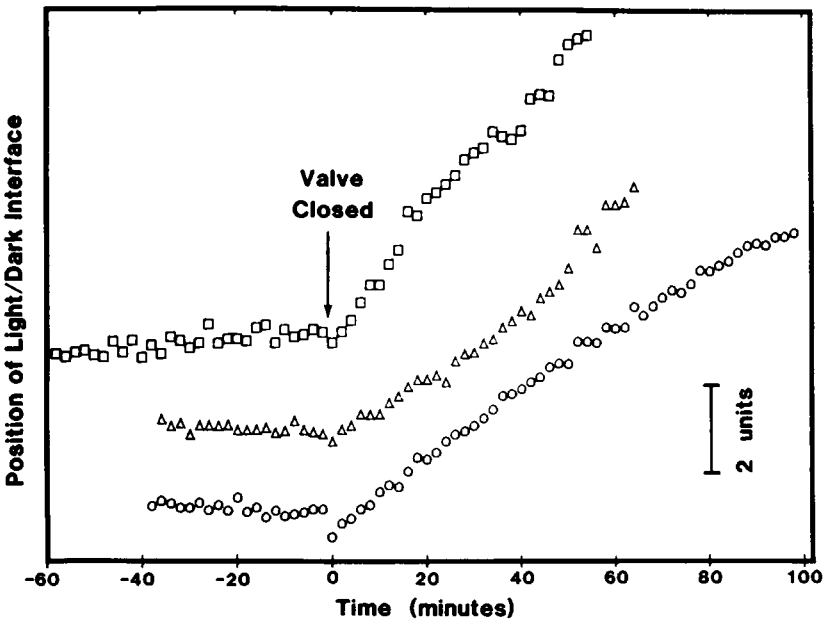

FIGURE 5 Control experiment. Position of the light/dark interface (in arbitrary units) vs. time for three RPE-choroid explants sealed in the chamber. Prior to time = 0 min, the pressure relief valve was open and the hydraulic permeability of the system very large. At time = 0, the pressure relief valve was closed (reducing the hydraulic permeability of the system to essentially zero) and the tissue began to bow forward. This experiment demonstrates that the tissue moved only in response to the hydrostatic gradient created by transepithelial fluid movement into the sealed half of the chamber. See text for details. As in Fig. 4, the three curves illustrated were separated vertically and the vertical scale is located in the *lower right* corner of the figure.

cells would have to swell at a rate sufficient to increase their thickness by a factor of 10 per hour for there to be an increase in cell thickness equivalent to the rate of tissue bowing. Because we could make measurements of the spontaneous net *trans*-RPE fluid movement for as long as 6 h, and the cells would burst before they obtained diameters of several hundred microns, clearly RPE edema was an insignificant source of tissue movement. Therefore, the tissue bowing in the chamber described in this report appears to be a response to RPE-mediated transepithelial fluid movement, exclusively.

Presumably, a hydrostatic gradient is necessary to force the tissue to bow forward in our apparatus. At time zero, the hydrostatic gradient was zero and increased with time. Fig. 4 shows that, in spite of this increasing hydrostatic gradient, the measured rate of fluid movement remained constant for an hour. This indicates that the pressure gradient is sufficiently small, at least for the first hour, so that it does not affect measurements of transepithelial volume change. Opening the pressure relief valve at the end of several experiments caused the tissue to fall backwards, thus verifying that this gradient exists. Presently, we have no means of quantifying this extremely small hydrostatic gradient.

The results that we report here corroborate the direction of RPE-mediated fluid movement reported previously (2, 7, 8, 11) and the rate is about the same as that measured by other groups using bullfrog RPE. Miller et al. (4, 7) have prepared bullfrog RPE-choroid explants with 50% greater transepithelial electrical resistance (and proportionally higher spontaneous transepithelial voltage) than our RPE-choroid explants. Apparently they were able to create a better electrical seal around the tissue in the Ussing chamber. They reported greater measured rates of fluid movement in preparations with higher electrical resistance (7). For reasons outlined earlier, it is possible that these previously reported values are reduced by tissue movement within the experimental chamber. It is conceivable then, that the technique reported here may yield greater measured rates of net *trans*-RPE fluid movement as we improve our tissue preparations. This technique seems to be applicable to any epithelium that can be dissected free of rigid supporting structures.

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