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Perspective

Consensus Recommendations for Studies of Outflow Facility and Intraocular Pressure Regulation Using Ex Vivo Perfusion Approaches

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Intraocular pressure (IOP) elevation is the primary risk factor and currently the main treatable factor for progression of glaucomatous optic neuropathy. In addition to direct clinical and living animal in vivo studies, ex vivo perfusion of anterior segments and whole eyes is a key technique for studying conventional outflow function as it is responsible for IOP regulation. We present well-tested experimental details, protocols, considerations, advantages, and limitations of several ex vivo model systems for studying IOP regulation. These include: (1) perfused whole globes, (2) stationary anterior segment organ culture, (3) perfused human anterior segment organ culture, (4) perfused animal anterior segment organ culture, (5) perfused human corneal rims, and (6) perfused human anterior segment wedges. These methods, with due consideration paid to their strengths and limitations, comprise a set of very strong tools for extending our understanding of IOP regulation.

Keywords: intraocular pressure regulation models, perfused anterior segment organ culture, ex vivo aqueous humor outflow regulation models

Glaucoma is a family of common optic neuropathies, often progressing to blindness and affecting well over 70 million people worldwide.[1–3] Elevated intraocular pressure (IOP) is the primary risk factor for most types of glaucoma and the major currently treatable factor for all forms, including normal tension glaucoma.[1,3,4] Various drug, laser, and surgical approaches are currently used to lower and control IOP with the goal of limiting progression of retinal ganglion cell loss.[3,5] Continued investigations to understand IOP regulation in normal and glaucomatous eyes, as well as the efficacy and optimization of various therapeutic approaches to controlling IOP, remain a central focus of vision research.

IOP depends jointly on aqueous humor (AH) inflow, produced by the ciliary body, and by the resistance to AH outflow.[1,4] Although there are intricate regulatory aspects of AH production,[6] except at very high pressures AH production is fairly independent of IOP.[7,8] The consequence of this is that regulation of the flow resistance to AH outflow is the main controller of IOP.[4,9,10] There are two primary aqueous outflow pathways: (1) the conventional pathway, which is through the trabecular meshwork (TM), Schlemm's canal inner wall endothelium (SCE), collector channels, and the aqueous drainage vessels; and (2) the uveoscleral or unconventional pathway through the ciliary muscle bundles.[3,4,11] Most of the outflow in humans is through the conventional pathway, which is pressure sensitive, but several other animal species have different proportions of conventional and uveoscleral outflow.[12] Most of the conventional outflow resistance resides in the deepest part of the TM and SCE, but a significant portion is also located beyond Schlemm's canal.[4,9,10,13–18]

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In the early 1980s, Polansky and colleagues[19–22] were successful in establishing primary cultures of TM cells, and, in 1998, Stamer and colleagues[23] isolated SCE cells. This allowed for biochemical and molecular analysis of these cultured cells. While their in vitro experiments provided a wealth of knowledge regarding TM and SCE cell biology, the establishment of primary cells in culture often causes the loss of some physiological properties. Additionally, because they are typically grown in an isolated state, these cell cultures are not easily amenable to studying flow, making it difficult to assess how modified cellular activity affects IOP. Perhaps more importantly, TM and SCE cells in vivo are in contact with other cells and tissue components that are not easily mimicked in cell culture, although studies on this topic are in process. A recent manuscript containing the consensus recommendations for TM cell culture provides considerable information and strong guidance for this approach.[24]

It is also noteworthy that several in vivo models, including mouse, non-human primate, canine,[25,26] and others, have also been used to provide valuable insights into IOP regulation.[27–30] The advantages of in vivo models over the ex vivo models discussed here include the intactness of the whole outflow system. The disadvantages include the differences between humans and animals, which are significant, and the difficulty in isolating responses and interactions of portions of the system without confounding influences. A recent manuscript containing the consensus recommendations for in vivo models provides considerable information and strong guidance for this approach.[27]

Caveats, Pros, and Cons

To study AH outflow and the regulation of IOP, several models, primarily ex vivo, have been developed. These models are clearly reductionist in nature, only providing information about portions of IOP regulation and the AH outflow pathway. Because they are ex vivo models, they have no AH inflow from the ciliary body, they have no active uveoscleral outflow for structural reasons, the episcleral veinous pressure is approximately 0 mm Hg, and the ocular pulse is absent. However, studies of the ocular pulse have been conducted with some of these models as will be discussed later.[31–36] The ciliary muscle is compromised without serum perfusion, and neural connections to the TM, ciliary muscle, and distal vessels are also compromised. Some aspects of the downstream contribution to the outflow resistance can and have been studied, but caution about the physiologic state of this region in these ex vivo methods must be carefully considered. Downstream cells can be isolated.[35] This has advantages, in that IOP regulation is complex and ex vivo models allow focused studies of the TM and SCE contributions to IOP regulation without the confounding effects of other portions of the system. However, it should be clear that the ciliary body, ciliary muscle, uveoscleral pathway, and components downstream of the SCE also make significant contributions to IOP regulation.[35,37,38]

Aqueous Humor Outflow

Steady-state AH dynamics are described by the modified Goldmann equation:[39]

$$F_{in} - F_u = C\,(IOP - EVP)$$

where aqueous production rate is F_{in}, unconventional or uveoscleral drainage rate is F_u, outflow facility is C, and episcleral venous pressure is EVP. In most ex vivo perfusions, the EVP is 0 mm Hg and there is no uveoscleral outflow, so that Goldmann's equation simplifies to

$$C = F_{in}/IOP$$

In ex vivo perfusion systems, F_{in} is the perfusion flow rate, either from the ciliary body or in this case from the perfusion, and IOP is the perfusion pressure in the anterior chamber. The flow rates are generally measured in nanoliters per minute (nL/min) or microliters per minute (µL/min), and the pressure is measured in millimeters of mercury (mm Hg). Results are often given as outflow facility, C, in nL/min/mm Hg or µL/min/mm Hg. They can also be presented as outflow resistance, which is the numerical inverse of outflow facility. Typical measured C values for in vivo human eyes are often somewhere around 0.29 µL/min/mm Hg depending on the measurement method and patient age.[40]

Here, we discuss the several ex vivo approaches most commonly used to study AH outflow regulation and IOP homeostasis, including the following:

1. Perfusion of enucleated whole globes
2. Stationary human anterior segment organ culture
3. Perfused human anterior segment organ culture
4. Perfusion of anterior segments from several other species
5. Perfusion of human corneas removed from transplant
6. Perfusion of wedges of human anterior segments.

Perfusion of Intact Enucleated Eyes

Quantitative perfusion studies of enucleated human eyes were conducted in the 1880s and fluid injections were conducted in 1863.[16] More detailed studies were then reported by several investigators, including Grant and Barany.[14–16,41–45] More recently, a number of studies using similar methods have been conducted perfusing intact human or bovine eyes.[46–49]

Typically, a small needle (e.g., 26- or 27-gauge) is inserted intracamerally through the peripheral cornea into the anterior chamber, carefully threading it through the pupil and then into the posterior chamber to prevent deepening of the anterior chamber, which can lead to artificial facility increases.[46] The needle insertion through the cornea is usually initially parallel to the corneal surface and it is then turned to go perpendicular to this surface. This produces an entry that is much less prone to leakage. Perfusion fluid is delivered from a reservoir suspended a defined distance above the eye or in some cases injected with controlled delivery rates by a syringe pump. The perfusion medium, commonly phosphate-buffered saline (PBS), was initially maintained at a constant pressure, with the flow rate determined gravimetrically as described by Grant.[14–16] Figure 1 shows a modified version of this system, which is often used for perfusion studies of whole eyes.

Whole eye perfusion has the advantage of being relatively simple, measuring facility across short time frames (hours) and not requiring extensive instrumentation. It is generally limited to eyes obtained within a few hours postmortem (ideally <<24 hours) and they can only be perfused for hours before postmortem changes in outflow properties become apparent.[50] Traditionally and most typically for

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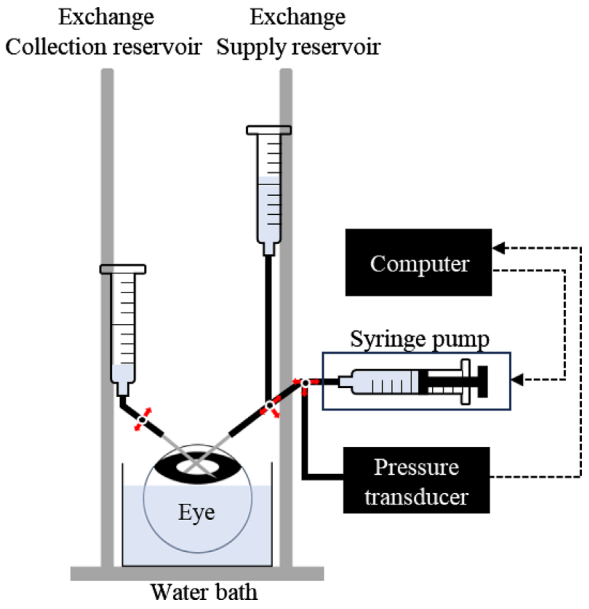

Figure 1. Schematic diagram illustrating the experimental setup for perfusion of intact enucleated eyes. The eye, immersed in a water bath, is connected to both an exchange supply reservoir and an exchange collection reservoir. During fluid exchange, the exchange supply reservoir delivers perfusion medium to the eye, and the exchange collection reservoir collects the exchanged fluid. When the connections between the reservoirs and the eye are closed, the perfusion medium is continuously delivered to the eye by the computerized syringe pump. Simultaneously, the flow rate and eye pressure are monitored for measuring outflow facility. *Solid black lines* represent fluid connections, *dashed black lines* represent electrical connections, and *red arrows* denote two- or three-way stopcocks.

humans, flow rates at 7 to 10 mm Hg perfusion head are in the range of 2.5 to 3 µL/min, which matches earlier studies by Brubaker.[7,8,51,52] This, of course, does not include episcleral venous pressure, as this is absent in enucleated eyes. Grant found normal enucleated human eyes to have outflow facilities averaging 0.17 at room temperature, which was equivalent to 0.22 at body temperature, and glaucomatous eyes ranged from 0.05 to 0.47 µL/min/mm Hg.[16] Individual variation was quite high and numerous others have conducted similar studies.[53]

Stationary Human Anterior Segment Organ Culture

To circumvent the relatively short time period that whole globes could be studied ex vivo, a stationary human anterior segment organ culture model was developed.[50,53] Rohen et al.[54] had earlier done similar short-term stationary organ culture studies measuring glycosaminoglycan biosynthesis.

Preparation and Dissection

Using aseptic technique in a cleaned and unobstructed laminar flow biosafety cabinet, the globe is rinsed with sterile PBS or culture medium (see below). Sometimes the external surface of the eye is rinsed with Betadine (10% povidone-iodine) to reduce possible contamination. The eye is then approximately bisected with an initial razor blade or scalpel cut followed by sterile small curved surgical scissors. Instruments can be washed and then gas autoclaved or rinsed extensively in 70% alcohol. The cornea, TM, and approximately 5 to 10 mm of sclera are used. The lens, iris, and ciliary body are gently teased away using Dumont forceps without damaging the TM. (If the dissection is too rough, the TM can be compromised.) The anterior segment is rinsed several times with sterile PBS or culture medium and placed in a well of a six-well culture dish with the anterior segment concave side up. The culture media should completely cover the anterior segment. Note that Figure 2 shows a similar dissection. Most investigators carefully remove all pigmented ciliary body remnants, as co-culture of the ciliary body with the TM causes signal transduction-related changes.

Culture Media and Incubation

Although other variants are occasionally used, Dulbecco's modified Eagle's medium (DMEM) containing 1× antibiotic-antimycotic (final concentration 100 units/mL penicillin G, 100 mg/ml streptomycin sulfate, and 0.25 mg/mL fungizone [amphotericin]), sterile and prewarmed to 25° to 37° C, is the most common approach. Note that anterior segment organ culture is normally conducted serum free, as AH contains almost no serum proteins. Adding serum will cause signaling changes and can also initiate TM cell migration off the beams, as is sometimes used to start TM cell cultures.[24] Anterior segments in these six-well plates are incubated in a sterile CO_2 incubator at 5% CO_2, 37°C, and 100% humidity to avoid evaporation, which could be significant; otherwise, the media are changed every 2 to 3 days. The TM tissue is very susceptible to damage by reduced pH, indicated by yellowing of the media, which occurs if the media is not changed frequently enough.[50] Other media can be used. Because glucose levels can change some cell signaling parameters, some studies mix high-glucose (4.5 mg/mL) and low-glucose (1 mg/mL) DMEM at a 1:1 ratio, whereas others use only high glucose. Clearly, changing glucose levels during experiments causes significant confounding signaling changes, so doing so should be avoided. There is no consensus on using high glucose or the 1:1 mixture. TM cells are very sensitive to glucose levels that are too high. Humidity maintained at 100% is critical to avoid evaporation, which would be problematic, and is usually achieved by adding a pan of water with 5% Betadine to the floor of the incubator.

Validation and Characterization

To initially characterize this method, the glycosaminoglycan (GAG) biosynthetic profile, cell viability, and ultrastructure in transmission electron micrographs (TEMs) were compared at different postmortem times with or without stationary culture.[50] Morphology by light microscopy and cell vitality were recovered nicely by days 3 to 5 in culture and maintained for well over 1 month in culture. The ultrastructure of the outflow pathway of uncultured tissue showed some deterioration with increased vacuoles and extracellular matrix disorganization by 24 hours postmortem and continued to worsen over time compared to fresh 2-to 12-hour postmortem tissue. However, tissue subjected to stationary culture retained good ultrastructural organization for 21 days in culture which was only slightly worsened by 28 days.[50] In addition, tissue placed in culture at 24, 48,

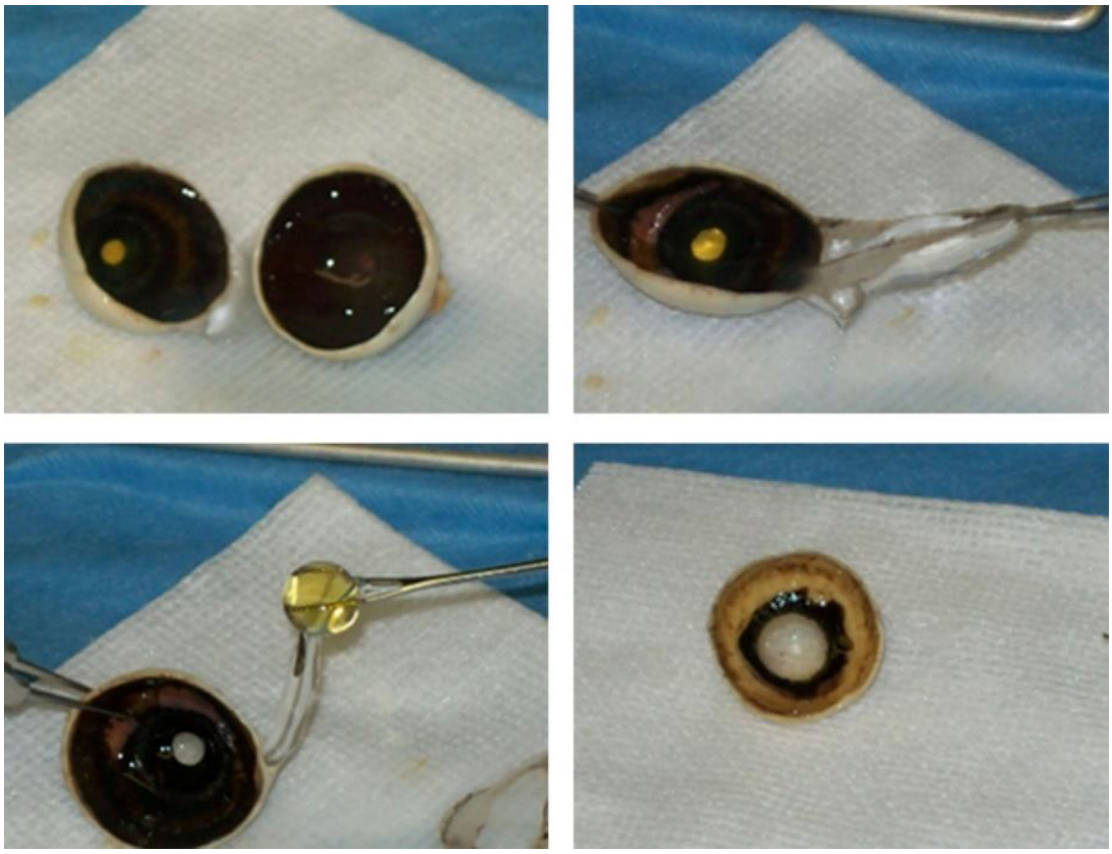

Figure 2. Dissection and preparation of human anterior segments for stationary or perfusion culture. Most investigators now carefully remove all the dark material seen covering the outflow pathway in the lower right panel without damaging the outflow pathway.

or even 72 hours postmortem, when eyes had been stored refrigerated and humidified after enucleation, regained good ultrastructure after stationary culture for around 5 days. The GAG biosynthetic profile behaved similarly. Such findings facilitate the use of ocular tissue that is obtained at longer times postmortem. Because of this observation, unless tissue is obtained within around 12 hours postmortem, it may be advisable to "recover" it in stationary culture for around 5 days before initiating most types of studies or before using it for perfusion culture as detailed in the next section.[50] This suggestion is not universally applied but does seem advisable.

Stationary organ culture is a viable tool for many kinds of studies where flow does not have to be taken into consideration.[50,55–58] As noted, the tissue and cells remain typical and functional for up to 21 to 28 days.[50] This also has some advantages because tissue integrity and organization are maintained. Different cell types of the outflow tissue (up to 19) have been identified using single-cell transcriptomics[59,60] and have presumably retained most of their relationships and interactions. Thus, this stationary culture approach has some significant advantages over cell culture for certain types of analyses.

Perfused Human Anterior Segment Organ Culture

Stationary human anterior segment organ culture was modified by Johnson et al.[61] and separately by Erickson-Lamy et al.[62] to develop a perfused organ culture method. This allows direct studies of outflow regulation and parameters for up to several weeks while facility is assessed continuously as impacted by various perturbations or conditions.[61,63,64] Here, we break down the overall perfusion culture setup and experimentation into separate steps for clarity.

Preparation of Human Donor Eyes

Eyes are procured by eye banks or hospital mortuaries within 24 to 48 hours postmortem. All excess muscle, orbital fat, and conjunctiva are removed from the external eye. Following brief sterilization by rinsing in Betadine (10% povidone-iodine) and sterile PBS, a superficial incision can be made around the limbus to release vascular resistance created by episcleral veins. Figure 2 shows photographs of the steps in the dissection process. Some researchers have determined that this initial superficial incision is not necessary. The whole eyes are bisected at the equator with a scalpel or single-edged razor blade, leaving enough sclera to secure to the flow cell (approximately 8–10 mm). The vitreous, retina, pars plana of the ciliary body, and lens are carefully removed. The iris and pars plicata are also normally removed to minimize shedding of pigment. The anterior segment is then washed with sterile PBS. Different groups carefully remove more or less of the iris/ciliary body because pigment can obstruct outflow. As a serum perfused tissue in vivo, it will deteriorate in serum-free conditions, and the ciliary body contains factors that affect aqueous outflow facility.

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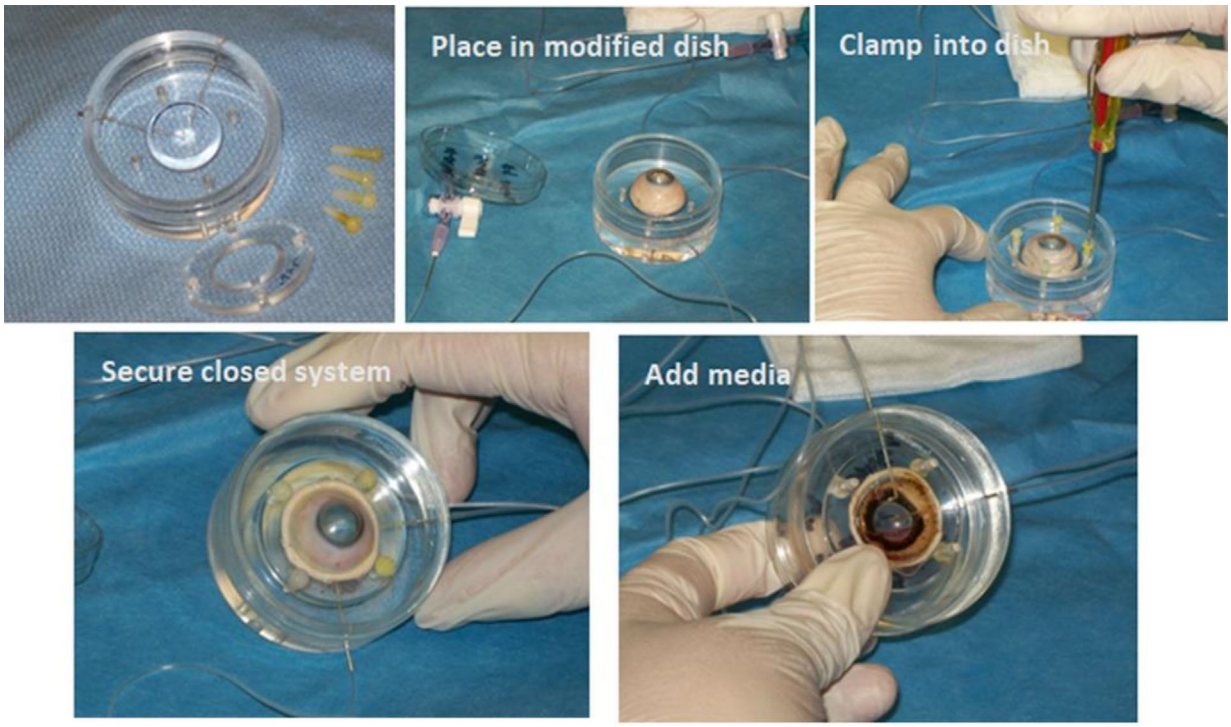

Figure 3. Setup mounting eye on flow cell.

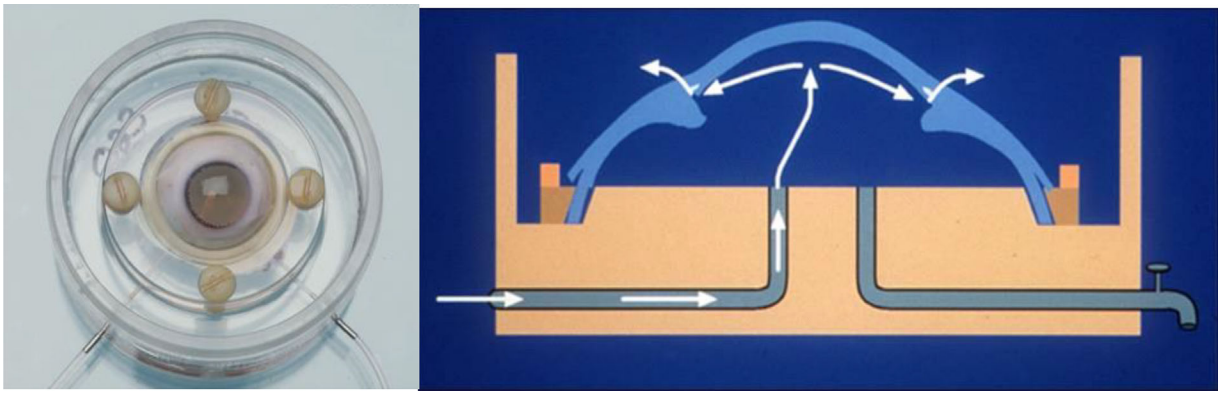

Figure 4. Perfusion cell with eye mounted. Top view of a flow cell with human anterior segment mounted. A cross-sectional diagram showing fluid routes and anterior segment placement and clamping. This is the Johnson flow cell design based on a machined, modified Petri dish.[61]

Perfusion Culture Setup

The dissected anterior segment is positioned and clamped on a perfusion cell forming a tight seal (Fig. 3). This perfusion cell is a modified petri dish flow cell (Fig. 4). The anterior segment is secured on the central pedestal with a circular sealing ring and screws, creating a closed chamber that can be perfused with media at the chosen flow rate (Fig. 5). The anterior segment is carefully centered on the pedestal, and the sealing ring gently applied over it, contacting the scleral rim of the anterior segment but not damaging the cornea or underlying outflow pathway. The mounting ring screws are tightened in a sequential/cross manner starting at position 1 with partially tightening, moving to position 3 with partially tightening, moving to position 2 with partially tightening, and then moving to position 4 with partially tightening. This is repeated for several cycles to achieve tight and uniform sealing of the tissue and avoid leaks. Occasionally after initial perfusion, additional tightening is needed. Some groups include a rubber O-ring over the sclera and beneath the clamping ring to further reduce leaks. Perfusion through the entry port is then started via a pump (Fig. 5) or a pressure head, as discussed later, and pressure transducer readings are initiated. Figure 6 shows a typical total system setup with pressure transducers, perfusion pump, and incubator. The perfusion can be constant flow, where a precise syringe pump provides adjustable flow at the desired rates (typically around 2.5 µL/min for human eyes or as needed) and a pressure transducer is used to obtain dynamic pressure readings. Alternatively, as detailed later, a constant perfusion

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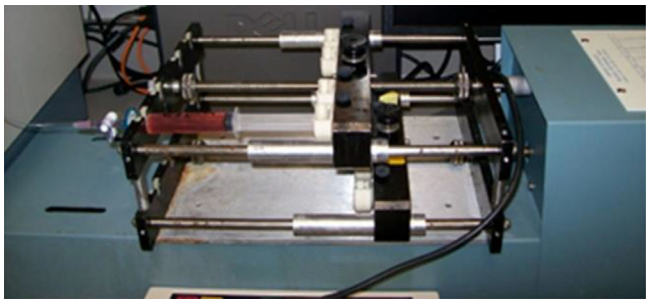

Figure 5. Constant flow syringe pump setup. An adjustable precision syringe pump can be used to produce low constant-volume perfusion. The flow rate can be adjusted as desired for the experimental design. Note that all syringe pumps have some misalignment of the lead screws, so that even a "constant flow" perfusion has some unsteadiness in the flow waveform due to the misalignment-induced oscillation of the pusher plate that contacts the syringe plungers. It is prudent to characterize the extent of pressure oscillation for each pump, such as by perfusing a fixed resistance such as a filter and measuring the resultant pressure waveform, which will be proportional to the flow waveform produced by the pump.

Figure 6. Perfusion system in the Fautsch Laboratory at Mayo Clinic (Rochester, MN). Shown is a humidified, 37°C, 5% CO_2 incubator with PE60 tubing connecting the flow chambers to the syringe pump (*blue box*) and to a pressure transducer (on the right outside the chamber). It is notable that a tray/pan of water with antibacterial such as Lysol placed in the bottom of each incubator and changed regularly is essential to maintain 100% humidity and to avoid evaporation errors. The monitor indicates pressure transducer readings.

head can be applied and fluid flow measured gravimetrically or by other methods.

Data Collection

For constant flow perfusion, pressure is measured by a pressure transducer attached to the second canula port on the dish the eye is mounted on. The pressure transducer (e.g., DTX Plus; Merit Medical, Galway, Ireland) is linked to an analog-to-digital conversion unit. Software then collects voltage data at regular intervals (e.g., once per minute) and averages readings of 60 time points to provide a one-hour readout. The collected data are converted, with suitable and regular calibration of the pressure transducers, into graphs of pressures, such as IOP (anterior chamber pressure) versus time. Regular calibration of the pressure transducers, possibly using a simple manometric technique, is important. The measurement time frame here is, of course, totally dependent on the type and rate of changes expected and experimental design considerations.

Post-Experiment Analysis

At the end of the experiment, the effects of treatments or manipulations on the TM can be assessed by dissecting the tissue for western or transcript analysis or cutting sections for immunohistochemistry, electron microscopy, or light microscopy to assess experimental tissue changes.

Post-Experiment Validation of Tissue Cellularity and Integrity

It is well known that a certain percentage of human eyes, particularly those from aged individuals, are compromised in one way or another.[65,66] Although the tissue often flows typically, in some cases physiological flow responses are not observed. Because there is no easy way to independently assess the condition of the outflow pathway tissue prior to or during the perfusion experiment, post-experiment assessment is absolutely required. Small radial tissue sections are always collected from different quadrants of each eye and placed in 4% paraformaldehyde or formalin for morphology and cellularity assessment. Hematoxylin and eosin (H&E) or toluidine blue staining allows determination of tissue organization and cellularity, both of which can be disrupted in some eyes.[65,66] This is a critical step, as it is not unusual for this post-analysis to show that there were few or even absolutely no TM cells in the outflow pathway or that the tissue is dramatically disrupted, sometime by earlier clinical glaucoma surgeries and sometimes just by the aging process. This happens frequently enough that all eyes must be assessed in this manner post-experimentation. Studies with these dramatic disruptions or cell loss must be thrown out as they are invalid. It is not unusual for an outflow pathway that is totally or nearly acellular on post-analysis to show typical baseline outflow facilities.[65,66] Judgment of the tissue condition requires some experience and entails comparison to normal tissue. Tissue initially destined for clinical transplant often has endothelial cell density, and when this is available it can be informative.

Criteria for Acceptance of Tissue and Results

Several criteria need to be considered before accepting, using, or interpreting data from human or other species tissue. Some of these criteria are experiment specific and some are generally necessary. Most eye-bank eyes come with some basic information, such as age, gender, race, cause of death, time of death, and time from death to enucleation, as well as other medical information often including some ocular disease information and medications. In general, at least some of this information is required, usually in a supplementary table, to publish studies based on these eyes.

Postmortem Time. Ideally, human eyes should be obtained within 12 hours postmortem and used immediately. Also quite important is time from death to enucleation; beyond 8 hours this can become problematic. In

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general, <12 hours postmortem is not practical for logistical reasons and most investigators use eyes out to either 24 or 48 hours postmortem.[65] Donor eyes that can be used for clinical transplant generally upstage research usage in terms of priority and expense. We have shown that, by 48 hours, some tissue changes are apparent, and these become quite pronounced by 72 hours.[50] As mentioned in the last section, stationary organ culture of human anterior segments for around 5 days restores morphology, ultrastructure, and glycosaminoglycan biosynthesis profiles to approximate eyes from 2 to 6 hours postmortem.[50] Because of this observation, some groups normally accept eyes out to 72 hours postmortem; if they are more than 12+ hours postmortem, they are subjected to stationary organ culture for around 5 days to "recover" them from postmortem effects. This practice is not universally used. When tissue is immediately placed in perfusion culture, unnaturally high outflow facilities are often observed and maintained for days.

Confounding or Unsafe Conditions. The medical information that commonly comes with eyes provides several indicators of acceptability of the eyes for the experiments planned. Research eyes are seldom tested for various diseases, so it is incumbent on the researcher and personnel using the eyes to assume that the donor had some severe infectious disease that was not listed on the eye-bank paperwork. Subsequently, eye preparation should always be conducted in a Biosafety Level 2 (BSL-2) safety cabinet using gloves, lab coats, goggles, and protocols that protect the dissector from transmission of possible unidentified diseases. Caution with dissecting instruments is also always a must because the transmittal of bloodborne or fluidborne diseases such as HIV/AIDS by a slip or accident must be avoided. Tissue remains should always be disinfected by soaking in 10% bleach and then disposed of in hazard bags.

For many types of studies, glaucoma surgeries such as trabeculectomy or minimally invasive glaucoma surgery (MIGS) in the donor eyes will compromise the outcomes. This is not always listed on the eye paperwork and occasionally is not known until the eye is dissected. Many aged donor eyes have also had cataract surgery, which per se is not an issue. The donors of glaucomatous eyes will often have been on one or more IOP control drugs, and this can impact the outflow pathway behavior in some cases. Because most glaucoma drugs are short acting, some of their effects may be dissipated by the time studies are conducted. Many are also focused on inflow or uveoscleral outflow and thus not primarily on the TM itself.

Depending on the type of study being conducted, other conditions such as cancer chemotherapy or other types of systemic treatments could impact interpretation of treatment efficacy or outflow facility. Sepsis, inflammation, or other types of medical situations can also impact facility or other types of studies. Although the outflow tissue is avascular and generally not highly available to the immune system, this is not absolute. Immune and vascular cells can be found in the outflow pathway.[59,60] Generally, these types of possible outflow effectors should be noted, and deviations from normal may require that results not be included. This is always a tricky call.

Age. Although using younger tissue would provide more robust study conditions, the average age of most eye-bank eyes for research is around 75+ years. Younger eyes are often used for corneal transplants. To the extent possible, age matching is important in experimental design. Age as an experimental variable is very difficult to consider due to the low proportion of eyes available from people less than 50 years old. Glaucoma is also a disease of aging.

Gender. Although no clear sex differences in outflow regulation are well established, it would not be surprising if there were some. Recent studies have highlighted intriguing estrogen dysregulation effects.[67] Funding agencies now require attention to this issue based on the long history of studies in men while ignoring women. Eye-bank eye distributions are often approximately equal in this regard. However, total eye availability is quite limited such that robust gender accountability is difficult to superimpose on the basic study proposed in almost all cases.

Actual outflow facilities for donor eyes can vary considerably, and some criteria for acceptance and rejection of outliers are valuable. Johnson et al.[68] showed that anterior segments with extremely low outflow rates, <1 µL/min, were often morphologically and ultrastructurally compromised. However, stationary cultured anterior segments were relatively normal.[50,68] For constant pressure perfusion at 8.8 mm Hg, anterior segments that flow much less than 1 µL/min or greater than 10 µL/min after a stabilization period (24–48 hours) are likely better excluded. For constant flow perfusion at 2.5 µL/min, anterior segments that have facilities less than 0.1 or more than 1 or 1.2 µLmin/mm Hg are usually not used. Limiting untreated eyes to this range, except for glaucomatous eyes, seems like a reasonable approach to eliminate extreme outliers.

Glaucoma. Criteria for designation of glaucoma eyes are not simple. Because IOP is a risk factor and not a simple cause for glaucoma and because normal-tension glaucoma is common, there are no universally accepted criteria. In addition, most anterior segment studies are focused on IOP regulation, not on glaucomatous nerve damage itself. Eye-bank eye sheets frequently list glaucoma and/or glaucoma drugs or treatments, and this is often taken as strong support for the eyes having glaucoma. Human eye-bank eyes are not considered human-subject research by the National Institutes of Health; however, obtaining full patient records from an ophthalmologist is a daunting task, requiring intense and ongoing human subject compliance and approvals. IOP records would be ideal, except the patient is immediately subjected to drug or other therapies to restore IOP control so only pretreatment values are of value. Other ophthalmic information, such as optical coherence tomography (OCT) scans, that documents the extent of damage can provide information about glaucoma progression. Eye-bank glaucomatous eyes are typically from patients that had glaucoma for 10 to 40 years and received various treatments throughout that time. Some investigators have an ophthalmologist grade the optic nerves of the putative glaucoma eyes they use, but this is not as simple as it might appear for tissue acquired 24 to 72 hours postmortem. This is also limiting, because this may have little to do with IOP regulation at the anterior chamber and will rely on the extent of damage acquired and the resilience of the individual optic nerve rather than the existence of IOP dysregulation. If clinical IOP control was in place early on, damage may be minimal and essentially undetectable in the postmortem tissue or in clinical records. One criterion would be to subject the anterior segment to a 2× pressure challenge, which glaucomatous eyes should fail (discussed later), but this is not always used.[9,69–71] In summary, there are no simple criteria, and none of the options mentioned above is even slightly

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adequate. This remains an unsettled point of controversy in the field, with occasional arbitrary criteria imposed. At the least, it is important to include all available information on the eyes and a comparison of facility for the glaucomatous and normal comparators in an appendix.

Troubleshooting

Leaks. In terms of very high flow rates, this is often an indication that the outflow pathway was damaged during the removal of the ciliary body/iris, etc. Another potential reason for high flow rates or facilities is the possibility of leaks around the anterior segment, at the tubing connections, or in the perfusion cell itself. These types of leaks are usually visible in terms of where perfusion fluid accumulates. A paper towel beneath the flow cell will make the latter types of leaks more noticeable. Some investigators add fluorescein to help visualize leaks. Leaks around the sealing ring can often be eliminated by additional tightening the nuts that hold the sealing ring or by reseating the anterior segment on the pedestal and reclamping it. This can only be done a few times, as the tissue becomes damaged and can no longer be clamped to create a seal. The rubber O-ring under the sealing ring suggested earlier could also help with this issue. Cutting the anterior segment with too little sclera will also result in leakage. It requires some experience to learn how tightly to clamp the sclera to get a good seal without extensive damage or cutting the tissue with the sealing ring edges.

Contamination. Flow cells should be washed with soap and rinsed extensively after each usage. Small passages (i.e., the perfusion ports) should also be rinsed extensively. If they are made of plastics that are not vulnerable to crazing upon exposure to 70% ethanol (e.g., polyvinyl chloride [PVC]), then the flow cells should be soaked and rinsed with 70% ethanol in the laminar flow biosafety cabinet after they have been washed with soap and rinsed thoroughly. Some groups use gas sterilization, which also works very well. Washing the biosafety cabinet with 70% ethanol before use is always advised, as well. Tubing and syringe pumps or perfusion head vessels should be cleaned similarly with soap, rinsed, and then rinsed again with 70% ethanol or gas sterilized. Autoclaving is also an option for components that can take the heat. If extensive rinsing afterward is conducted, 5% bleach solutions can also be used. The same is true for the dissecting tools.

Because eye tissue is not collected under sterile conditions, rinsing extensively prior to opening the eye is always advisable. Removal of muscle and other external tissue also diminishes contamination. Here, a Betadine wash can be helpful, although extended exposure to the cornea can compromise the ion pumps and cause it to swell. At any sign of contamination throughout the process, throw the eye away. It is never viable to try to clean it, and all solutions and components should be cleaned and/or discarded.

Solutions should also be sterile. The CO_2 incubator and biosafety cabinets also should be extensively and completely cleaned periodically. High-efficiency particulate air (HEPA) filters in the biosafety cabinets should be tested regularly, usually every 6 months or yearly. HEPA filters on the CO_2 incubator, if available, should be changed regularly, and the culture room must be cleaned and dust removed occasionally. Keeping covers over all flow cells, tissue, etc., when moving between the biosafety cabinet and the incubator is critical. Keeping covers on the flow cell inside the incubator and keeping the door closed as much as possible can also reduce contamination. Contamination is a much larger issue than in cell culture, as you are starting with a nonsterile tissue and have more points of entry of contaminants. In terms of the antibiotic/antimycotic, this should be maintained as frozen aliquots and stability is only a few days at 37°C. The antimycotic stock is a suspension, rather than a solution, so it should be mixed well before adding to medium. Repeated freeze/thaw is also not advisable.

Culture Room Pressure. If you are doing minimal biohazard experiments, it is advantageous to have the culture room pressure positive. This will push air out slightly and minimize incoming bacterial, fungal, yeast, or viral contamination from adjacent labs or rooms. On the other hand, if you are doing serious biohazard work, such as viral or such infections, you should have the room at a negative pressure to avoid escape of biohazard agents to adjacent labs or rooms. This is normally a requirement of biosafety committees. Because most culture rooms are not HEPA filtered, incoming air cannot be assumed to be contaminant free and people walk in and out the doors.

Experimental Design and Power Calculations

To the extent possible, paired-eye studies have more power, as biological variation between individuals is almost always larger than left–right eye differences in an individual. When feasible, using an eye as its own internal control is advisable (i.e., before and after a treatment). Normally, facility is quite consistent over days to a few weeks in perfusion culture, making this a viable approach.

Although the variability compared to the size of the treatment effect expected is always somewhat difficult to predict, unless numerous very similar studies have been previously conducted, power calculation to determine sample sizes is usually quite important. This is also increasingly emphasized by funding agencies. The biological variation between individuals, particularly older human eye donors, is typically quite high. The good news is that, in most cases, the biological variability is effectively "noise" and does not include the variables of interest in most studies. However, this must be considered in any power calculations.

From the observed variability in numerous studies over many years, most studies with medium-sized effects require a sample size of five or six human eye pairs to achieve clear statistical resolution in paired-eye or pre/post internal control designs studies. Because the size of the effect expected can be quite different for different types of experiments, some adjustments to this may be essential. For very subtle differences between treatment and control, sample sizes as large as seven to 10 pairs may be necessary.

A caveat to the sample sizes mentioned above is the occasional eye or pair of eyes that, upon postmortem evaluation of H&E sections, exhibit either very low cellularity or major tissue disorganization. These eyes are typically just thrown out as invalid and not included in the sample size. It is of note, however, that older eyes exhibit significant TM cell loss and this is much accentuated in aged glaucomatous eyes, as the loss can be as much as 50%.[72–75] Because TM cellularity is an important component of glaucomatous outflow compromise, this must be considered carefully within the context of the experimental design.

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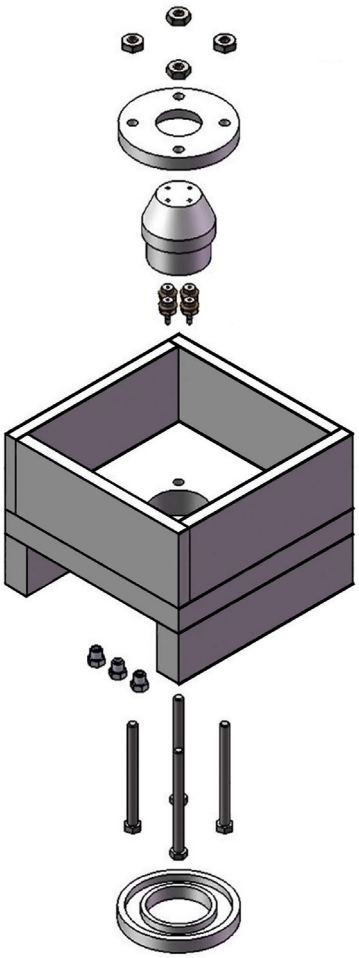

Figure 7. Exploded view of a modification of the Johnson and the Erickson-Lamy flow cells. There are four ports and flow channels through the pedestal with tubing nipples on the bottom. Unused ports can be filled with the included small plastic bolts, taking them out of play. This flow cell is machined from PVC, which is relatively resistant to solvent and heat exposure, and glued together or bonded by dissolving the two surfaces to be joined with a solvent such as butyl methacrylate or by acrylic cement. This flow cell is available through Apex Industries, Inc. (Tigard, OR; www/apexind.com; A-008309 Rev B01, Square Eye Cell Assembly–Clear PVC). Recently, for convenience, this cell has been flipped over and placed cornea side down on a small water glass for perfusion instead of cornea side up as is the traditional method. When used right-side up, as in the diagram, a sterile aluminum foil top is used to avoid contamination.

Variations in Perfused Human Anterior Segment Organ Culture

An alternative flow cell design is shown in Figure 7. As with the original flow cells of Johnson et al. or Erickson-Lamy et al., this cell can be used with constant flow and pressure transducer measurement of resultant pressure generated or it can be used with constant pressure perfusion and flow rate measured gravimetrically (Fig. 8).

For perfusion at constant flow, a pressure transducer is connected to one port, as detailed earlier. For perfusion at constant pressure, either a 100-mL culture bottle or a 50-mL conical centrifuge tube can serve as the perfusion reservoir filled with perfusion medium and positioned such that the fluid level is the desired distance above the flow cell sealing ring (as in Fig. 8), which is where the perfused fluid seeps out of the episcleral veins. A perfusion head of 8 or 8.8 mm Hg (10.8761 or 11.9637 cmH_2O) is considered 1× and is equivalent to perfusion at 2.5 to 3 µL/min, which is typical for normal human eyes excluding episcleral venous pressure.[52] Tygon tubing is attached to one perfusion nipple on the bottom of the flow cell and to a broken-off 1-mL plastic pipette inserted through a hole in a loose-fitting lid on the perfusion bottle. Alternatively, the Tygon tubing is inserted through a small hole high up in the side of the centrifuge tube with the cap loosely attached. It is preferrable to use tubing with a small inner diameter with a moderately rigid wall so that there is less dead space and less tubing compliance (tubing expansion or shrinking with pressure changes). Also, it is important to remove all air bubbles from the system. Using two-way stop cocks for connections facilitates this. The other perfusion ports on the bottom of this flow cell are sealed off when not in use but can be used for drug or agent delivery without interrupting flow (described later).

Constant pressure gravimetric flow measurements can be long term, where the tubing is clamped with a hemostat, removed from the bottle, and the bottle weighed on a balance at time intervals of greater than 15 minutes and often only a few times a day. In this case, three readings 15 minutes apart are averaged to reduce reading errors. The bottle must be handled with gloves to avoid moisture on the hands modifying the weight. Often, the changes in outflow facility are slow, as in the case of IOP homeostatic responses or other treatments, and this long-term measurement protocol is advantageous.

For shorter term or more frequent gravimetric measurements, microbalances can be placed in humidified (100% humidity) CO_2 incubators and continuous measurements passed through a USB-linkage and conversion box to be captured on a laptop in Excel files. As an example, two Ohaus AZ423/E Adventurer Precision balances were placed in each CO_2 incubator (Fig. 9) and continuously monitored via USB output passage through a GageMux USB 4-port universial gauge (ADS/QMS; Advanced Systems & Designs, Chagrin Falls, OH, USA) interface to a laptop as an Excel file (Fig. 10). The GageMux can be set to read each of the four balances in the two incubators every 2 minutes or at longer intervals.

Gravimetric measurements can be somewhat noisy because the desired flow rate is obtained by numerically differentiating the measured weight versus time. Vibrations and direct air currents such as created by the incubator fans should also be carefully avoided, especially if weights are taken over relatively short time intervals so that differences are small. With precautions, this is generally not an issue. Of course, many alternative setups are viable. The Excel file can then be processed to produce flow (µL/min) or facility output (C = flow/pressure) as a function of time and integrating various treatments into the profile.

Steel Eye Facility Measurements

Ethier et al.[76] developed a "steel eye" perfusion method (Fig. 11). This method is relatively easy and inexpensive to set up and produces excellent data. The central idea is that flow rate is determined by measuring the pressure drop across a fixed known resistor (resistance tubing). After calibration, it is relatively easy to convert the pressure drop into a flow rate, and this approach does not suffer from some of the noise that can affect gravimetric methods. Complete

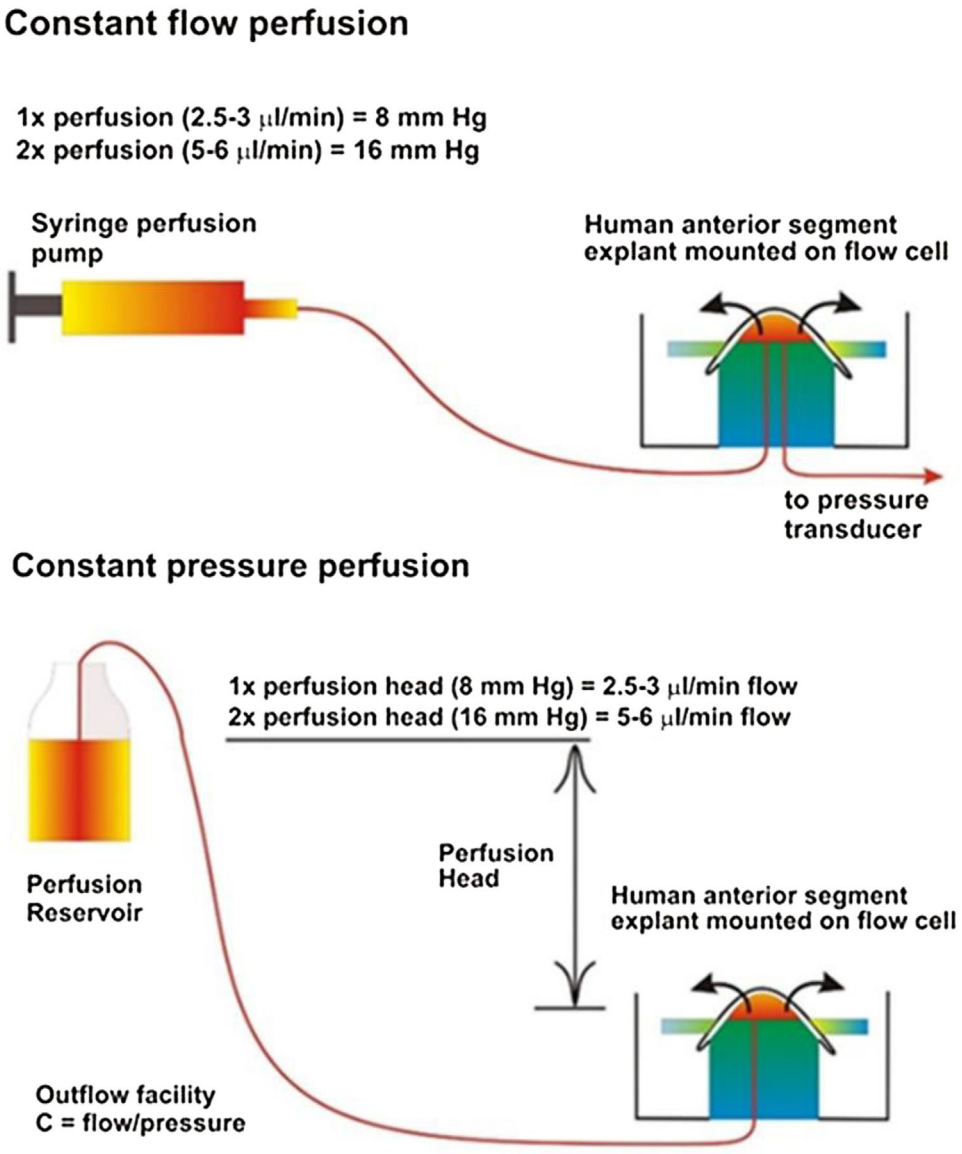

Figure 8. Two approaches to determining outflow facility: constant flow and constant pressure perfusion. The two methods use either constant perfusion rates to measure the resultant pressure with a pressure transducer or constant pressure to measure flow rate, usually gravimetrically. Both methods have advantages.

details are provided in their manuscript.[76] Although shown perfusing a whole eye, it is easily adapted to use with the flow cells as discussed above. The reference flow resistance is provided by a length of 30-gauge tubing (0.15-mm inner diameter and 60 cm long from Vita Needle Company, Neeham, MA, USA). A pressure reservoir is positioned at a pressure head above the eye or flow cell (Pres). The actual pressure at the eye (IOP) is Pres minus the pressure drop across the resistance tubing (DP), which is read by the pressure transducer (DPT). The flow resistance tubing is calibrated, and both pressure transducers are separately calibrated. Fluid outflow rate through the eye at a given IOP is then calculated from the DP reading and previous calibration values. A computer-controlled feedback loop can be incorporated to adjust the reservoir height such that the pressure delivered to the eye remains constant even if flow rate (and hence pressure drop across the resistance tubing) changes.

Flow Meters

Flow meters, which measure flow in the ranges required here, are another option. This approach works well and at significantly lower cost, although calibration is a potential issue to be dealt with. There are commercially available microfluidics flow rate sensors that directly measure the flow rates in the range of 1 to 50 µL/min, but with proper calibration and handling they can reach sensitivities in the 5-nL/min range.[77]

iPerfusion System

Sherwood et al.[77–80] developed a high-precision flow measurement system, the iPerfusion system. This system is well designed, stable, and precise and has been detailed previously so is not discussed further here.

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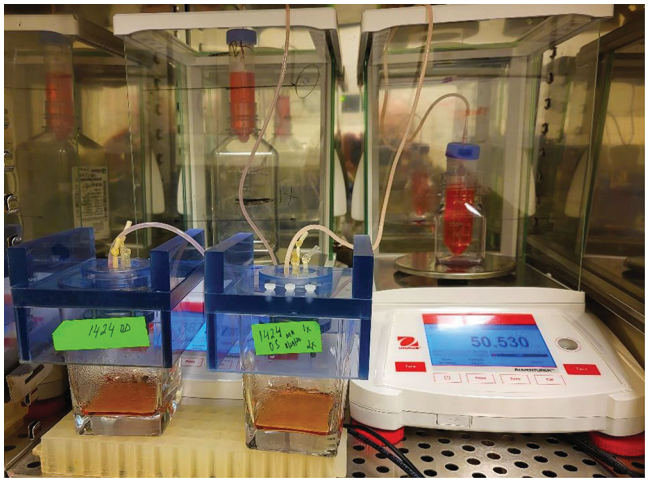

Figure 9. Balances and flow cells for continuous gravimetric assessment of flow in a constant pressure perfusion approach. Two balances weigh fluid in 50-mL conical centrifuge tubes with flow cells inverted on a water glass (*lower left*). *Left* is at 2× and *right* is at 1× pressure. Balances are constantly read at 2-minute intervals and data are inserted into an Excel file.

Figure 10. GageMux USB with foot pedal to initiate readings and four USB connections to read the four balances. Data are inserted into an Excel file on a connected laptop.

Drug, Treatment, Modulators, or Agent Delivery

Of course, one important aspect of these model systems is studying the effects of various molecular or cellular modulators or drugs on outflow facility. Several approaches are commonly used.

Push–Pull Syringes. To deliver agents to the anterior chamber during an experiment without changing the pressure on the outflow pathway, two extra ports on a four-port flow cell (Fig. 7) can be attached to a pair of syringes mounted on a push–pull device. This can also be done by disconnecting and substituting connections if only two ports are available. The two syringe barrels are attached to a board or rod facing away from each other and at a distance such that one plunger is completely depressed and the other is just barely into the syringe. The two plungers are clamped to each other such that pushing them fills one while emptying the other. One is filled with the delivery solution and then both are attached to different ports on the flow cell. Slowly ejecting fluid from the filled syringe into the flow cell simultaneously sucks an equal volume of fluid out of the flow cell. If this is done slowly (i.e., over a few minutes), the anterior chamber fluid is exchanged for the treatment fluid without changing the pressure in the anterior chamber. It is usually advisable to clamp off or close the other port or ports to avoid mixing during this process. If the syringe volume is somewhat larger than the anterior chamber volume in the flow cell, a full or even over 100% replacement of perfusion fluid can be achieved. If the treatment is to be continued, the fluid in the syringe and connecting tubing of a constant flow system or the fluid in the connecting tubing and perfusion bottle must be changed. Several variations on this approach can be used, including a two-reservoir gravity/pressure differential fluid exchange approach.

In-line Injection. Sometimes when small volumes are to be delivered, the agent can be injected into the delivery Tygon tubing using a small (e.g., 27-gauge) needle. This is complicated by the fact that the agent will be diluted during this process and the exact dose at the time it hits the outflow pathway is very difficult to predict. However, for some types of treatment where it is the amount of agent and not the concentration that is important or the treatment is continued over longer times, this can be suitable. New Tygon tubing will take this injection without leaking, but if it has been used several times and has become more rigid, leaks can occur, interfering with facility measurements. Vacuum grease can also be applied to the hole afterward to minimize leaks. If larger volumes are to be injected over short time periods, it will be difficult not to increase the pressure head transiently.

Direct Injection. In some cases, it is possible to inject the agent directly through the central cornea and into the anterior chamber. The injection with a small needle (again, 27-gauge or smaller) and using the parallel–perpendicular approach as detailed in the whole eye perfusion section above can deliver the agent close to the outflow pathway, although this relies on diffusion of the agent at rates faster than the fluid flow to avoid non-uniform delivery to different quadrants of the anterior chamber.

Dismount and Process. Of course, some agents, treatments, surgeries, etc., cannot be injected but will require dismounting the anterior segment, conducting the procedure, and then remounting it on the flow cell. The sealing O-ring mentioned above is particularly helpful in this situation.

Outflow Segmentation and Soaked-In Versus Flowed-In. The increased awareness of the degree of outflow segmentation—the observation that outflow is not uniform around the circumference of the eye but occurs in regions where there is high, intermediate, and low or no flow—may significantly complicate agent delivery to the outflow pathway. This is true for both experimental and clinical delivery. As can be seen with perfusion of flow markers (discussed later), there are numerous regions scattered around the eye that exhibit very different levels of flow.[9,81,82] The high-flow regions of a normal human anterior segment

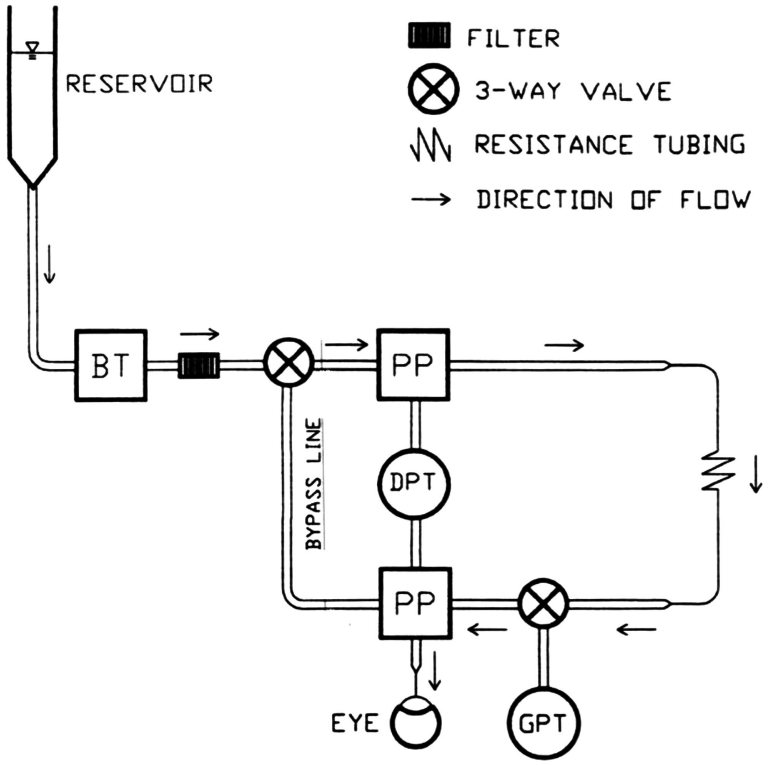

Figure 11. Improved outflow measurement device developed by Ethier et al.[76] The reservoir is mounted on a screw drive to adjust the head. BT indicates a bubble trap, and PP indicates bubble purge ports. DPT is the pressure transducer that reads pressure across the steel tube, and GPT is a pressure transducer that reads equivalent to the IOP at the eye. *Arrows* show the direction of flow during a measurement.

comprise approximately 1/3 of a human anterior segment and probably account for 70% to 80% of the total flow, with the intermediate regions making up around 20% to 30% of the total flow and the low-flow regions accounting for almost none of the total flow. The highly segmental flow marker distributions after a 1-hour perfusion tell us that delivery of large agents will be similarly segmental, with the low-flow regions being essentially not treated at all and the high-flow regions receiving nearly all of the agent. However, small drugs or agents will have high diffusion coefficients, and the distances, even with the convoluted TM shape and pathway they must traverse, are relatively small (hundreds of µm). Thus, small agents at uniform concentrations in the AH or perfusion medium will likely achieve uniform delivery circumferentially within reasonably small times. Larger agents such as proteins, virus, nanoparticles, or antibodies, are more complex and time dependent. Large agents that soaked in with the flow stopped overnight show relatively uniform delivery to all segmental regions of the outflow pathway.[81,83–85] Thus, the delivery conditions may be important, and residency time of larger agents in the perfusion medium or anterior chamber could be critical. Consequently, it is probably incumbent on investigators to address this issue for medium to large agents or drugs.

Targeted Delivery to Low-Flow Regions. Ethier's group[86] developed a method of targeting stem cells to various regions of the outflow pathway independently of outflow segmentation. This involved using magnetic nanoparticles and magnet-guided delivery. This could be very useful for some types of studies alleviating the outflow segmentation patterns.

Perfusion of Anterior Segments From Other Species

Flow cells designed for human anterior segments are too small for bovine anterior segments but can be used with porcine and monkey anterior chambers.[86–90] For porcine anterior chambers, the sharp sticky side of four small Velcro strips or an O-ring can be placed on the inside of the clamping ring to keep the sclera from slipping out. The porcine

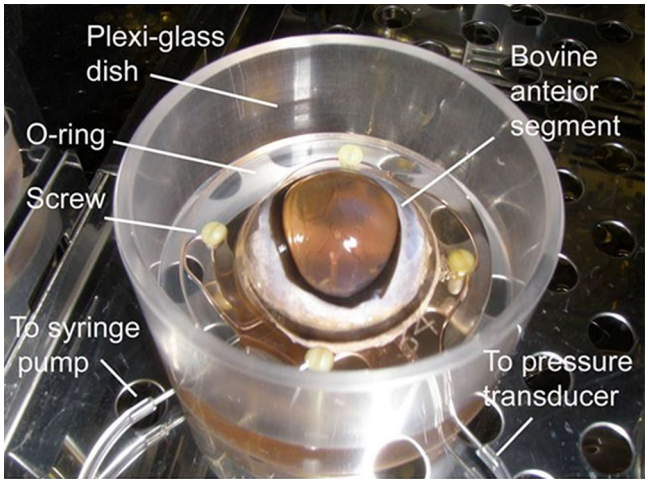

Figure 12. The bovine anterior segment perfusion culture model.[91] A perfusion cell similar to that shown in Figure 4 but scaled larger is shown with a bovine anterior segment attached.[91,92]

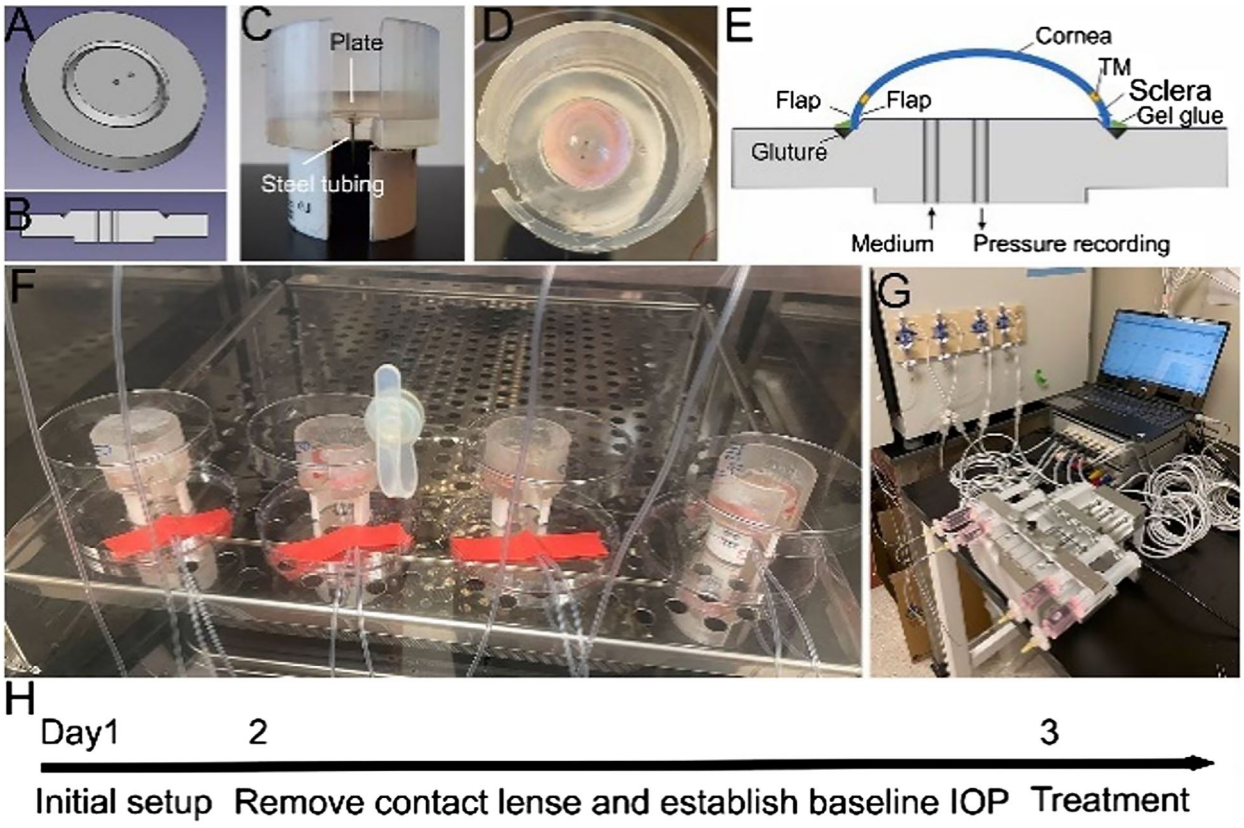

Figure 13. Corneal trephine transplant tissue perfusion culture model.[100] (**A**, **B**) The perfusion plate. (**C**) The perfusion plate and holder. (**D**) The corneal tissue glued onto the plate. (**E**) Side view of the corneal tissue after being glued onto the plate. (**F**, **G**) Perfusion system setup. (**H**) Experimental timeline. (Reprinted from Peng M, Margetts TJ, Sugali CK, et al. An ex vivo model of human corneal rim perfusion organ culture. *Exp Eye Res*. 2022;214:108891.)

sclera is much more pliable than in humans and is thus difficult to clamp. Some investigators use Super Glue (cyanoacrylate) to solve this problem. Several investigators have used bovine anterior segments successfully, as well, with modifications of the flow cell dimensions (Fig. 12).[91,92] Perfused bovine or porcine anterior chambers typically flow at somewhat faster rates than human, around 4.5 to 6 µL/min, compared to 2.5 to 3 µL/min for humans. Monkey organ-cultured anterior segment (MOCAS) cultures have also been used for anterior segment organ culture.[87] With proper attention to specifics, these animal anterior segments can serve as good substitutes for human anterior segments and are often much more available.

Considerations

Most species, except for human and mouse, exhibit washout, a gradual decrease in the outflow resistance per volume perfused.[43,46,48,92–97] This phenomenon requires special consideration when conducting perfusion studies. It can also be useful in investigating mechanisms, if handled carefully.[46,48,93] It is also notable that porcine corneas tend to swell during perfusion and can obstruct the outflow pathway after several days of culture. In general, 5 to 7 days is the upper limit that is usable without this being problematic. However, one group successfully extend this out to 21 days, although it is unclear how this was achieved.[98]

Corneal Transplant Tissue

Eye banks prepare tissue to be used for corneal transplant by cutting tissue slightly larger, often using a trephine, than the cornea and leaving a small amount of sclera and most of the outflow pathway. When used for transplantation, the central portion of the cornea is removed by a different trephine. This tissue before the cornea that is removed is occasionally available and is an appealing source of human anterior segments for perfusion studies. They are often less expensive and include better quality tissue from younger donors. They contain the cornea, a few millimeters of sclera, and usually most or all of the TM. The amount of sclera in corneal transplant tissue is often not sufficient when using standard flow cells. If the corneal tissue is clamped in the Johnson flow cell, the IOP will often be very high (sometimes over 50 mm Hg), which is likely due to the TM being pulled toward the dish caused by mechanical strain. As a solution to this problem, Mao et al.[99,100] developed a modified three-dimensional printed flow cell where these anterior segments can be glued into place to achieve a seal (Fig. 13). Instead of clamping, a perfusion plate was designed and the corneal tissue glued on the plate using cyanoacrylate glues. These glues are easy to apply, polymerize rapidly, and provide reliable attachment and sealing between the corneal tissue and the perfusion plate.[100]

Procedure

The corneal tissue is first cleaned of residual uveal tract tissues. The scleral tissue is scored at the inner and outer surface posterior to the limbal area to prevent glue from entering the TM. After drying the edge of the corneal tissue (sclera), the corneal tissue is glued to the perfusion plate using the thin cyanoacrylate glue (Gluture). This step is not intended to seal the corneal tissue but rather to provide initial attachment for the next step. After glue polymerization, the corneal tissue is securely attached to the plate. The cornea is then covered with a piece of contact lens, and a bead of caulk-like cyanoacrylate glue (Super Glue Gel Control) is applied around the base of the corneal tissue. The caulk-like glue provides a strong attachment and seal of the anterior chamber after incubation in a culture incubator to allow the caulk-like glue to cure. Because cyanoacrylate glue is moisture cured, the incubator should be at 100% humidity, which is always essential anyway to avoid evaporation and is maintained by a pan of water as discussed earlier. The flow cell with the corneal tissue glued in place is then connected with the perfusion system and perfused overnight (with the contact lens still on the cornea). The next day, the contact lens is carefully removed. The corneal tissue is further perfused for another day for IOP to stabilize at a baseline IOP.[100] The materials details, three-dimensional (3D) printing files, protocol specifics, and an instructional video are available and will not be reiterated here.[100] However, several key steps are emphasized and explained in more detail below.

Selection of Corneal Tissue. The corneal tissue should have relatively even with 2- to 3-mm sclera tissue all round. Sometimes there are notches or unevenness at the edge of the sclera tissue. Although the sclera can be trimmed to make it even, that process will further reduce the amount of sclera available for model setup. This will cause two problems: (1) the corneal tissue becomes too small to fit onto the plate (there is a groove designed for the edge of the corneal tissue), and (2) the glue is more likely to contaminate the TM, causing high baseline IOP.

Drying. The glues used in this method are moisture-curing glues. If the region to be glued contains water, the glue will polymerize immediately and will not provide proper attachment. It is recommended to use a cotton swab to carefully dry the region before applying the glue. Also, caution should be exercised not to dry the cornea or TM to prevent cell loss.

Glue Contamination in the TM. Glue contamination is not uncommon for inexperienced users. Glue contamination in the TM will increase TM stiffness and clog the outflow pathway, causing very high IOP (usually >40 mm Hg). There are a few tips to minimize the chance of glue contamination. First, apply a minimum amount of glue. Second, trim the edge of the corneal tissue. If the edge of the corneal tissue is not even, when it is placed on the perfusion plate the protruded region will lift the surrounding region, creating gaps or voids. To fill the gaps, more glue is needed, which increases the chance of glue contamination. Third, carefully score the sclera to create flaps. These flaps function like a roof with a drip edge,

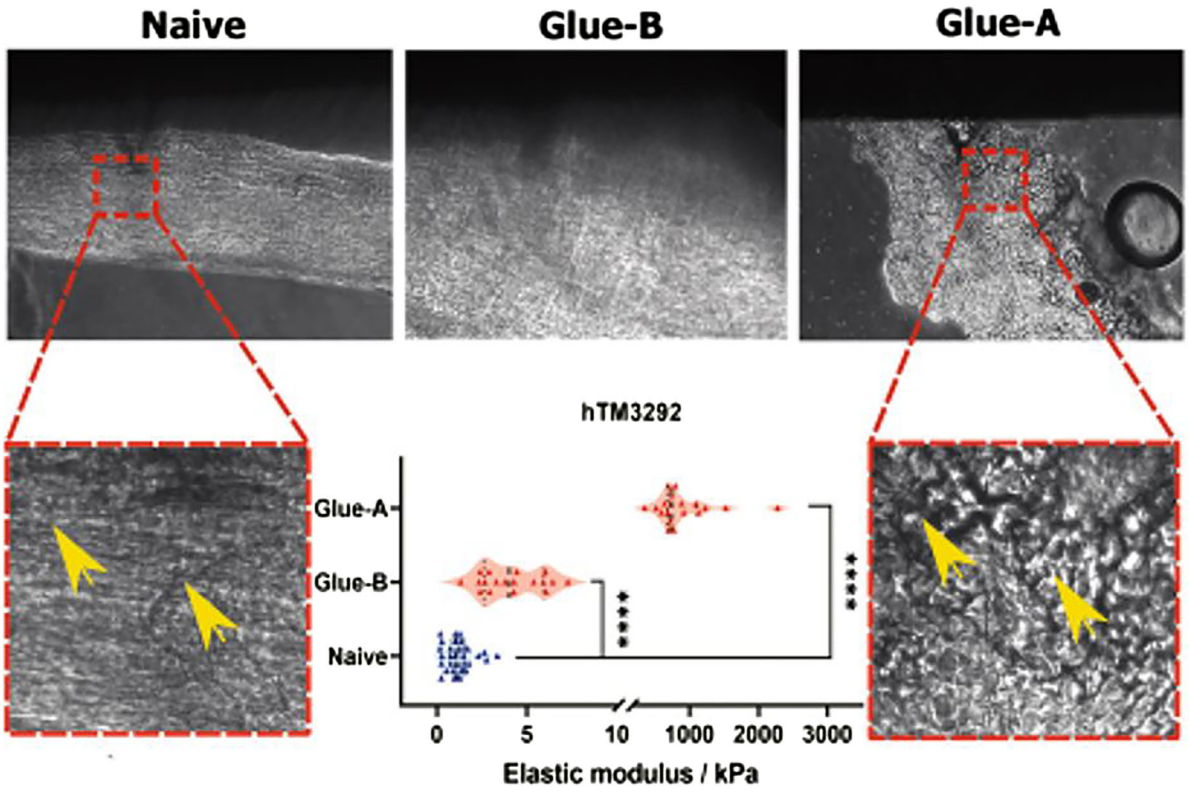

Figure 14. Glue contamination increased TM stiffness in perfusion cultured human corneal tissue.[100] The stiffness (elastic modulus) of three corneal tissues with no (naïve), mild/non-visible (Glue-B), and severe (Glue-A) glue contamination. (Reprinted from Peng M, Margetts TJ, Sugali CK, et al. An ex vivo model of human corneal rim perfusion organ culture. *Exp Eye Res*. 2022;214:108891.)

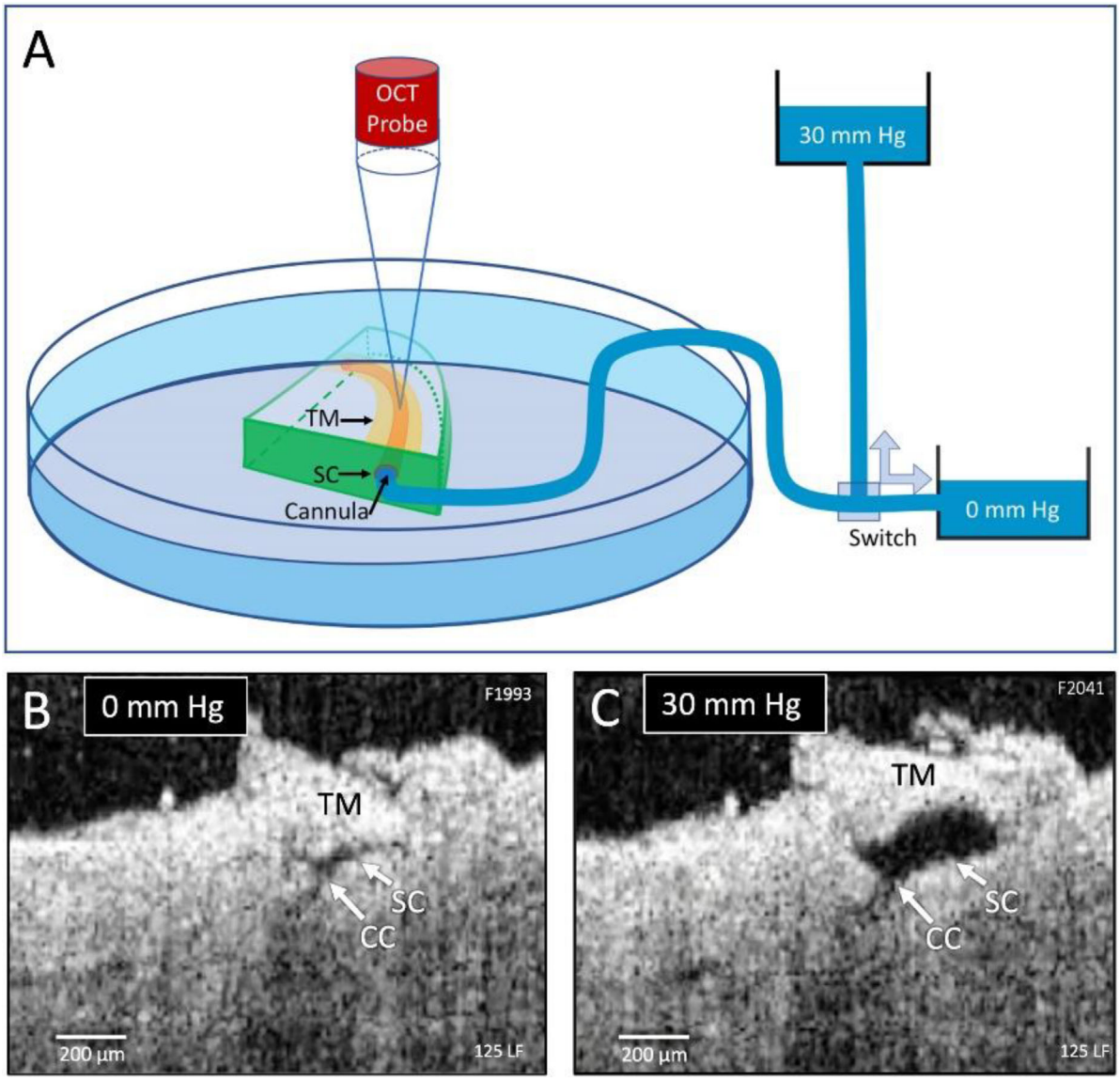

Figure 15. (**A**) The Johnstone model of anterior segment wedge with cannula in Schlemm's canal.[31] The positive pressure in Schlemm's canal is determined by the level of the pressure reservoir (0 or 30 mm Hg) relative to the fluid level in the dish. (**B**, **C**) OCT image at 0-mm Hg pressure head (**B**) and at 30-mm Hg positive pressure head (**C**). The pressure head can be varied rapidly to achieve dynamic responses. *Scale bar*: 200 µm. (Reprinted from Johnstone M, Xin C, Acott T, et al. Valve-like outflow system behavior with motion slowing in glaucoma eyes: findings using a minimally invasive glaucoma surgery-MIGS-like platform and optical coherence tomography imaging. *Front Med (Lausanne)*. 2022;9:815866.)

preventing glue from being siphoned into the TM or limbal region. Although this corneal tissue perfusion culture model has many advantages, it has several disadvantages as can be seen in Figure 14:

1. Inability to reposition during experiments—Because the corneal tissue is glued onto the perfusion plate, it cannot be repositioned.
2. Glue contamination cannot be removed during perfusion—When there is suspicion of glue contamination in the TM (very high IOP), there are no good methods to remove polymerized glue from the TM without causing tissue damage. For fixed corneal tissue after perfusion culture, glue can be removed by soaking in tissue clearing reagents such as xylene.
3. Post-perfusion tissue collection—Cyanoacrylate glue provides very strong attachment, and it is almost impossible to "tear" the tissue from the perfusion plate; therefore, the corneal tissue must be cut off using a sharp blade.

Wedge Flow and Pressure Models

Johnstone et al.[31,34] developed a novel model where human anterior segment wedges were cut and Schlemm's canal cannulated with a 130- to 150-µm outer diameter cannula and positive pressure heads applied to study the deformation of the SCE, juxtacanalicular connective tissue (JCT), and

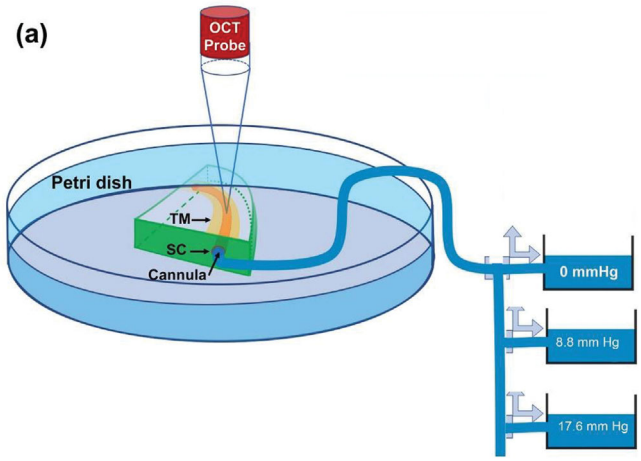

Figure 16. Wedge flow for negative Schlemm's canal pressure studies. The pressure head is from the surface of the fluid in the Petri dish to the surface of the fluid in the perfusion reservoir: 0 mm Hg, 8.8 mm Hg (1×), or 17.6 mm Hg (2×). The anterior chamber pressure is higher than the Schlemm's canal pressure as in the normal eye.

the TM beams in response to SC pressures (Fig. 15). This was used with OCT 3D imaging at different Schlemm's canal pressure heads, static and fluctuating, combined with inverse finite-element analysis to obtain biomechanical properties of normal and glaucomatous tissue as it was distended toward the anterior chamber.[31,34,101,102] As indicated in the figure, the pressure in Schlemm's canal was equal to or greater than that in the anterior chamber. This provides an excellent model to study the dynamics and biomechanical properties of the outflow tissue and impacts of the ocular pulse and coupling between outflow pathway components, SC, and episcleral vessels.

Karimi et al.[103] modified this model by using lower Schlemm's canal pressures relative to anterior chamber pressures (Fig. 16). The pressures were from the fluid level over the wedge in the Petri dish to the pressure head below this in the reservoirs at 8.8, 17.6, or 26.5 mm Hg. This is equivalent to the physiological 1×, 2×, and 3× pressures normally observed from the human anterior chamber to Schlemm's canal in vivo. This system, imaged with green visual OCT provides much higher 3D resolution of the TM. The OCT 3D images obtained from scanning systems in Figures 15 and 16 are amenable to inverse finite-element analysis with modeling elastin and different collagens or other extracellular matrix regions. Both approaches allow assessment of outflow pathway biomechanics using different parameters and pressures.

Outflow Segmentation Markers

Because AH outflow is very segmental, labeling regions of high, intermediate, and low flow or no flow allows determining molecular and cellular correlations to relative flow.[104–107] Several methods have been developed. Cationic ferritin was used earlier and at higher levels, which clogged the outflow system.[82,107] Derivatized quantum dots (QDots), fluorescent microspheres (FluoSpheres) with negative anionic or positive cationic charges, or membrane-permeant CellMasks have been used along with numerous other markers.[104–106,108] Because cell surfaces and extracellular matrix are generally negatively charged, cationic FluoSpheres tend to bind to these surfaces and be retained for weeks. However, there are enough of both types of charges that anionic FluoSpheres are also retained for weeks in the outflow pathway. Comparison of marker levels defines segmental regions for relevant molecular or cellular studies. Usually, labels are flowed in for approximately 1 hour at the end of an experiment. As a test for marker binding rather than relative flow regions, soaking the marker overnight without flow should provide uniform marker distribution, demonstrating that flow, not binding patterns, is being assessed. It is also useful to flow in one color marker at an initial time and then another color marker after a time interval to see whether the regions are dynamic or if treatments change their distributions.[108,109] Another point of interest is that atomic force microscopy (AFM) studies are incompatible with most FluoSphere-like markers and CellMask is necessary for these studies.

Recently, angiography has been utilized to identify high- and low-flow areas in porcine and human anterior segments.[110,111] This can be correlated with in vivo/clinical patterns. Ex vivo anterior segments are incorporated into a constant flow system.[77] Upstream from the perfusion chamber is a three-way stop cock that is an entry point for the tracer. Tracers include 2% Fluorescein (Akorn Pharmaceuticals, Lake Forest, IL, USA) or 0.4% indocyanine green (ICG; (Cardiogreen; Sigma-Aldrich, St. Louis, MO, USA). After a stable outflow facility baseline is achieved, 2 mL of the tracer is introduced into the eye via an upstream three-way stop cock and then exits the perfusion chamber to a downstream waste reservoir placed between the eye and pressure transducer open to atmosphere. After completion of the tracer introduction, the perfusion rate is continued at the original rate.

For this setup, the camera is a clinical SPECTRALIS HRA+OCT (Heidelberg Engineering, Heidelberg, Germany) that can image fluorescein and ICG, but it is instead installed on a FLEX module. This means that the SPECTRALIS camera head is detached from the manufacturer-provided table and installed on a manufacturer-provided boom arm to allow positioning of the camera head above and pointing down at the eye within the perfusion chamber. Typically, a 30° lens is used with a 25-diopter focus, although other lenses that have different working distances are available. The device software allows images to be taken as single acquisitions or short videos for either tracer with a timer. In one study,[110] after angiography the eye was dismounted and tracer rinsed out prior to subsequent experimental steps. Alternatively, residual tracer can also be flushed from the eye using the perfusion system perfusate similar to the original tracer delivery method.

IOP Homeostatic Responsiveness

The most significant indicator of anterior segment health and functional integrity is the ability to mount a physiological IOP homeostatic response to a 2× pressure challenge.[9,69,70] Normal anterior segments respond to a 2× pressure challenge by reducing the outflow resistance over the time period of a day or so in an attempt to restore pressure to its normal level.[9,69,70,112] This response is absent in glaucomatous anterior segments or in anterior segments depleted of TM cells by saponin treatment.[71,113] It can be restored by restoration of TM cellularity.[113] This test of anterior segment functionality is relatively easy to administer, in that doubling

the perfusion pressure or perfusion flow rate, which triggers a 2× pressure immediately, followed by sustaining the 2× pressure or flow rate for several days, initiates a reduction in the outflow resistance over this time period, if the anterior segment is functionally healthy.[9,69–71,113]

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References

1. Quigley HA. Glaucoma. *Lancet*. 2011;377:1367–1377.
2. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol*. 2006;90:262–267.
3. Weinreb RN, Leung CK, Crowston JG, et al. Primary open-angle glaucoma. *Nat Rev Dis Primers*. 2016;2:16067.
4. Stamer WD, Acott TS. Current understanding of conventional outflow dysfunction in glaucoma. *Curr Opin Ophthalmol*. 2012;23:135–143.
5. Weinreb RN, Aung T, Medeiros FA. The pathophysiology and treatment of glaucoma: a review. *JAMA*. 2014;311:1901–1911.
6. Delamere NA. Ciliary body and ciliary epithelium. *Adv Organ Biol*. 2005;10:127–148.
7. Brubaker RF. The measurement of pseudofacility and true facility by constant pressure perfusion in the normal rhesus monkey eye. *Invest Ophthalmol*. 1970;9:42–52.
8. Brubaker RF. The effect of intraocular pressure on conventional outflow resistance in the enucleated human eye. *Invest Ophthalmol*. 1975;14:286–292.
9. Acott TS, Vranka JA, Keller KE, Raghunathan V, Kelley MJ. Normal and glaucomatous outflow regulation. *Prog Retin Eye Res*. 2021;82:100897.
10. Johnson M, Erickson K. Mechanisms and routes of aqueous humor drainage. In: Albert D, Jakobiec F, eds. *Principles and Practice of Ophthalmology*. Philadelphia: W.B. Saunders; 2000:2577–2595.
11. Weinreb RN. Uveoscleral outflow: the other outflow pathway. *J Glaucoma*. 2000;9:343–345.
12. Kaufman PL. Pressure-dependent outflow. In: Ritch R, Shields MB, Krupin T, eds. *The Glaucomas*. St. Louis: Mosby; 1996:307–335.
13. Johnson M. What controls aqueous humour outflow resistance?. *Exp Eye Res*. 2006;82:545–557.
14. Grant WM. Facility of flow through the trabecular meshwork. *Arch Ophthalmol*. 1956;54:245–248.
15. Grant WM. Further studies on facility of flow through the trabecular meshwork. *Arch Ophthalmol*. 1958;60:523–533.
16. Grant WM. Experimental aqueous perfusion in enucleated human eyes. *Arch Ophthalmol*. 1963;69:783–801.
17. Rosenquist R, Epstein D, Melamed S, Johnson M, Grant WM. Outflow resistance of enucleated human eyes at two different perfusion pressures and different extents of trabeculotomy. *Curr Eye Res*. 1989;8:1233–1240.
18. Sit AJ, McLaren JW. Measurement of episcleral venous pressure. *Exp Eye Res*. 2011;93:291–298.
19. Polansky JR, Alvarado JA. Isolation and evaluation of target cells in glaucoma research: hormone receptors and drug responses. *Curr Eye Res*. 1985;4:267–279.
20. Polansky JR, Weinreb R, Alvarado JA. Studies on human trabecular cells propagated in vitro. *Vision Res*. 1981;21:155–160.
21. Polansky JR, Weinreb RN, Baxter JD, Alvarado J. Human trabecular cells. I. Establishment in tissue culture and growth characteristics. *Invest Ophthalmol Vis Sci*. 1979;18:1043–1049.
22. Polansky JR, Wood IS, Maglio MT, Alvarado JA. Trabecular meshwork cell culture in glaucoma research: evaluation of biological activity and structural properties of human trabecular cells in vitro. *Ophthalmology*. 1984;91:580–595.
23. Stamer WD, Roberts BC, Howell DN, Epstein DL. Isolation, culture, and characterization of endothelial cells from Schlemm's canal. *Invest Ophthalmol Vis Sci*. 1998;39:1804–1812.
24. Keller KE, Bhattacharya SK, Borras T, et al. Consensus recommendations for trabecular meshwork cell isolation, characterization and culture. *Exp Eye Res*. 2018;171:164–173.
25. Gelatt KN, Gum GG. Inheritance of primary glaucoma in the beagle. *Am J Vet Res*. 1981;42:1691–1693.
26. Gelatt KN, Gum GG, Gwin RM, Bromberg NM, Merideth RE, Samuelson DA. Primary open angle glaucoma: inherited primary open angle glaucoma in the beagle. *Am J Pathol*. 1981;102:292–295.
27. McDowell CM, Kizhatil K, Elliott MH, et al. Consensus recommendation for mouse models of ocular hypertension to study aqueous humor outflow and its mechanisms. *Invest Ophthalmol Vis Sci*. 2022;63:12.
28. Overby DR, Clark AF. Animal models of glucocorticoid-induced glaucoma. *Exp Eye Res*. 2015;141:15–22.
29. Rasmussen CA, Kaufman PL. Primate glaucoma models. *J Glaucoma*. 2005;14:311–314.

30. Kuchtey J, Olson LM, Rinkoski T, et al. Mapping of the disease locus and identification of ADAMTS10 as a candidate gene in a canine model of primary open angle glaucoma. *PLoS Genet*. 2011;7:e1001306.
31. Johnstone M, Xin C, Acott T, et al. Valve-like outflow system behavior with motion slowing in glaucoma eyes: findings using a minimally invasive glaucoma surgery-MIGS-like platform and optical coherence tomography imaging. *Front Med (Lausanne)*. 2022;9:815866.
32. Johnstone MA. The aqueous outflow system as a mechanical pump: evidence from examination of tissue and aqueous movement in human and non-human primates. *J Glaucoma*. 2004;13:421–438.
33. Johnstone MA, Saheb H, Ahmed II, Samuelson TW, Schieber AT, Toris CB. Effects of a Schlemm canal scaffold on collector channel ostia in human anterior segments. *Exp Eye Res*. 2014;119:70–76.
34. Wang K, Johnstone MA, Xin C, et al. Estimating human trabecular meshwork stiffness by numerical modeling and advanced OCT imaging. *Invest Ophthalmol Vis Sci*. 2017;58:4809–4817.
35. Roy Chowdhury UR, Fautsch MP. Isolation and culture of vascular distal outflow pathway (VDOP) cells from human donor eyes. *Curr Protoc*. 2022;2:e528.
36. Roy Chowdhury U, Bahler CK, Holman BH, Dosa PI, Fautsch MP. Ocular hypotensive effects of the ATP-sensitive potassium channel opener cromakalim in human and murine experimental model systems. *PLoS One*. 2015;10:e0141783.
37. Roy Chowdhury U, Hann CR, Stamer WD, Fautsch MP. Aqueous humor outflow: dynamics and disease. *Invest Ophthalmol Vis Sci*. 2015;56:2993–3003.
38. McDonnell F, Dismuke WM, Overby DR, Stamer WD. Pharmacological regulation of outflow resistance distal to Schlemm's canal. *Am J Physiol Cell Physiol*. 2018;315:C44–C51.
39. Goldmann H. The aqueous veins and the Poiseuille law. *Ophthalmologica*. 1949;118:496–519.
40. Kazemi A, McLaren JW, Lin SC, et al. Comparison of aqueous outflow facility measurement by pneumatonography and digital Schiotz tonography. *Invest Ophthalmol Vis Sci*. 2017;58:204–210.
41. Grant W, Trotter R. Tonographic measurements in enucleated eyes. *Arch Ophthalmol*. 1955;53:191–200.
42. Bárány E. In vitro studies of the resistance to flow through the angle of the anterior chamber. *Acta Soc Med Upsal*. 1953;59:260–276.
43. Bárány EH, Scotchbrook S. Influence of testicular hyaluronidase on the resistance to flow through the angle of the anterior chamber. *Acta Physiol Scand*. 1954;30:240–248.
44. Bárány EH, Woodin AM. Hyaluronic acid and hyaluronidase in the aqueous humour and the angle of the anterior chamber. *Acta Physiol Scand*. 1954;33:257–290.
45. Van Buskirk EM, Brant WM. Influence of temparature and the question of involvement of cellular metabolism in aqueous outflow. *Am J Ophthalmol*. 1974;77:565–572.
46. Overby D, Gong H, Qiu G, Freddo TF, Johnson M. The mechanism of increasing outflow facility during washout in the bovine eye. *Invest Ophthalmol Vis Sci*. 2002;43:3455–3464.
47. Gong H, Brown K, Johnson M, Kamm RD, Freddo TF. Hydraulic conductivity of juxtacanalicular connective tissue using quick-freeze/deep-etch. *Invest Ophthalmol Vis Sci*. 1997;38(suppl):S564.
48. Gong H, Freddo TF. The washout phenomenon in aqueous outflow–why does it matter? *Exp Eye Res*. 2009;88:729–737.
49. Gong H, Yang CY. Morphological and hydrodynamic correlations with increasing outflow facility by rho-kinase inhibitor Y-27632. *J Ocul Pharmacol Ther*. 2014;30:143–153.
50. Acott TS, Kingsley PD, Samples JR, Van Buskirk EM. Human trabecular meshwork organ culture: morphology and glycosaminoglycan synthesis. *Invest Ophthalmol Vis Sci*. 1988;29:90–100.
51. Brubaker R. Measurement of aqueous flow by fluorophotometry. In: Ritch R, Shields, MB, Krupin T, ed. *The Glaucomas*. St. Louis: Mosby; 1996:447–454.
52. Brubaker RF. Flow of aqueous humor in humans. *Invest Ophthalmol Vis Sci*. 1991;32:3145–3166.
53. Van Buskirk EM, Pond V, Rosenquist RC, Acott TS. Argon laser trabeculoplasty. Studies of mechanism of action. *Ophthalmology*. 1984;91:1005–1010.
54. Rohen JW, Schachtschabel DO, Lütjen-Drecoll E, Rohrbach M, Berghoff K, eds. *Morphological and Biochemical Studies on Cell and Tissue Cultures of Human Trabecular Meshwork*. Berlin: Springer-Verlag; 1983:39–43.
55. Acott TS, Nobis CA, Van Buskirk E. Studies of human trabecular extracellular proteoglycans. *Proc Int Soc Eye Res*. 1986;4(suppl.):184.
56. Acott TS, Samples JR, Bradley JMB, Bacon DR, Bylsma SS, Van Buskirk EM. Trabecular repopulation by anterior trabecular meshwork cells after laser trabeculoplasty. *Am J Ophthalmol*. 1989;107:1–6.
57. Acott TS, Truesdale AT, Samples JR, Van Buskirk EM. Trabecular extracellular matrix synthesis in human explant organ culture. *Invest Ophthalmol Vis Sci*. 1986;26(suppl.):211.
58. Acott TS, Westcott M, Passo MS, Van Buskirk EM. Trabecular meshwork glycosaminoglycans in human and cynomolgus monkey eye. *Invest Ophthalmol Vis Sci*. 1985;26:1320–1329.
59. van Zyl T, Yan W, McAdams A, et al. Cell atlas of aqueous humor outflow pathways in eyes of humans and four model species provides insight into glaucoma pathogenesis. *Proc Natl Acad Sci USA*. 2020;117:10339–10349.
60. Patel G, Fury W, Yang H, et al. Molecular taxonomy of human ocular outflow tissues defined by single-cell transcriptomics. *Proc Natl Acad Sci USA*. 2020;117:12856–12867.
61. Johnson DH, Tschumper RC. Human trabecular meshwork organ culture. *Invest Ophthalmol Vis Sci*. 1987;28:945–953.
62. Erickson-Lamy K. The perfused human ocular anterior segment as a model for aqueous outflow physiology. *J Glaucoma*. 1992;1:44–53.
63. Johnson DH, Tschumper RC. The effect of organ culture on human trabecular meshwork. *Exp Eye Res*. 1989;49:113–127.
64. Johnson DH, Bradley JM, Acott TS. The effect of dexamethasone on glycosaminoglycans of human trabecular meshwork in perfusion organ culture. *Invest Ophthalmol Vis Sci*. 1990;31:2568–2571.
65. Ethier CR. Perfusion organ culture of anterior segments: a twenty-year odyssey of practice and pitfalls. *Presented at the ARVO 2008 Annual Meeting*, Ft. Lauderdale, FL, April 27–May 1, 2008.
66. Fautsch MP, Johnson DH. Aqueous humor outflow: what do we know? Where will it lead us? *Invest Ophthalmol Vis Sci*. 2006;47:4181–4187.
67. Youngblood H, Schoenlein PV, Pasquale LR, Stamer WD, Liu Y. Estrogen dysregulation, intraocular pressure, and glaucoma risk. *Exp Eye Res*. 2023;237:109725.
68. Johnson DH. Human trabecular meshwork cell survival is dependent on perfusion rate. *Invest Ophthalmol Vis Sci*. 1996;37:1204–1208.

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69. Acott TS, Kelley MJ, Keller KE, et al. Intraocular pressure homeostasis: maintaining balance in a high-pressure environment. *J Ocul Pharmacol Ther*. 2014;30:94–101.
70. Acott TS, Kelley MJ, Keller KE, et al. IOP homeostasis - why most people do not ever develop glaucoma. In: Knepper PA, Samples JR, eds. *Glaucoma Research and Clinical Advances 2016–2018*. Amsterdam: Kugler Publications; 2016:87–98.
71. Raghunathan VK, Benoit J, Kasetti R, et al. Glaucomatous cell derived matrices differentially modulate non-glaucomatous trabecular meshwork cellular behavior. *Acta Biomater*. 2018;71:444–459.
72. Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals. *Ophthalmology*. 1984;91:564–579.
73. Alvarado J, Murphy C, Polansky J, Juster R. Age-related changes in trabecular meshwork cellularity. *Invest Ophthalmol Vis Sci*. 1981;21:714–727.
74. Grierson I, Calthorpe CM. Characteristics of meshwork cells and age changes in the outflow system of the eye: their relevance to primary open-angle glaucoma. In: Mills KB, ed. *Glaucoma: Proceedings of the Fourth International Symposium of the Northern Eye Institute*. Oxford: Pergamon Press; 1989:12–31.
75. Grierson I, Howes RC. Age-related depletion of the cell population in the human trabecular meshwork. *Eye (Lond)*. 1987;1(pt 2):204–210.
76. Ethier CR, Ajersch P, Pirog R. An improved ocular perfusion system. *Curr Eye Res*. 1993;12:765–770.
77. Sherwood JM, Reina-Torres E, Bertrand JA, Rowe B, Overby DR. Measurement of outflow facility using iPerfusion. *PLoS One*. 2016;11:e0150694.
78. Sherwood JM, Boazak EM, Feola AJ, Parker K, Ethier CR, Overby DR. Measurement of ocular compliance using iPerfusion. *Front Bioeng Biotechnol*. 2019;7:276.
79. Madekurozwa M, Reina-Torres E, Overby DR, Sherwood JM. Direct measurement of pressure-independent aqueous humour flow using iPerfusion. *Exp Eye Res*. 2017;162:129–138.
80. Madekurozwa M, Reina-Torres E, Overby DR, van Batenburg-Sherwood J. Measurement of postmortem outflow facility using iPerfusion. *Exp Eye Res*. 2022;220:109103.
81. Keller KE, Bradley JM, Vranka JA, Acott TS. Segmental versican expression in the trabecular meshwork and involvement in outflow facility. *Invest Ophthalmol Vis Sci*. 2011;52:5049–5057.
82. de Kater AW, Melamed S, Epstein DL. Patterns of aqueous humor outflow in glaucomatous and nonglaucomatous human eyes. A tracer study using cationized ferritin. *Arch Ophthalmol*. 1989;107:572–576.
83. Hann CR, Bahler CK, Johnson DH. Cationic ferritin and segmental flow through the trabecular meshwork. *Invest Ophthalmol Vis Sci*. 2005;46:1–7.
84. Swaminathan SS, Oh DJ, Kang MH, et al. Secreted protein acidic and rich in cysteine (SPARC)-null mice exhibit more uniform outflow. *Invest Ophthalmol Vis Sci*. 2013;54:2035–2047.
85. Swaminathan SS, Oh DJ, Kang MH, Rhee DJ. Aqueous outflow: segmental and distal flow. *J Cataract Refract Surg*. 2014;40:1263–1272.
86. Snider EJ, Kubelick KP, Tweed K, et al. Improving stem cell delivery to the trabecular meshwork using magnetic nanoparticles. *Sci Rep*. 2018;8:12251.
87. Hu Y, Gabelt BT, Kaufman PL. Monkey organ-cultured anterior segments: technique and response to H-7. *Exp Eye Res*. 2006;82:1100–1108.
88. Quinn RF, Tingey DP. Effects of cytoskeleton-reactive agents on aqueous outflow facility in a porcine ocular anterior segment preparation. *Can J Ophthalmol*. 1995;30:4–10.
89. Goldwich A, Ethier CR, Tamm ER. Perfusion with the olfactomedin domain of myocilin does not show major effects on outflow facility in anterior eye segment organ cultures. *Invest Ophthalmol Vis Sci*. 2002;43:1036.
90. Snider EJ, Hardie BA, Li Y, et al. A porcine organ-culture glaucoma model mimicking trabecular meshwork damage using oxidative stress. *Invest Ophthalmol Vis Sci*. 2021;62:18.
91. Mao W, Tovar-Vidales T, Yorio T, Wordinger RJ, Clark AF. Perfusion-cultured bovine anterior segments as an ex vivo model for studying glucocorticoid-induced ocular hypertension and glaucoma. *Invest Ophthalmol Vis Sci*. 2011;52:8068–8075.
92. Erickson-Lamy K, Rohen JW, Grant WM. Outflow facility studies in the perfused bovine aqueous outflow pathways. *Current Eye Research*. 1988;7:799–807.
93. Kelly RA, McDonnell FS, De Ieso ML, Overby DR, Stamer WD. Pressure clamping during ocular perfusions drives nitric oxide-mediated washout. *Invest Ophthalmol Vis Sci*. 2023;64:36.
94. Erickson-Lamy K, Schroeder AM, Bassett-Chu S, Epstein DL. Absence of time-dependent facility increase ("washout") in the perfused enucleated human eye. *Invest Ophthalmol Vis Sci*. 1990;31:2384–2388.
95. Kaufman PL, True-Gabelt B, Erickson-Lamy KA. Time-dependence of perfusion outflow facility in the cynomolgus monkey. *Curr Eye Res*. 1988;7:721–726.
96. Ramos RF, Stamer WD. Effects of cyclic intraocular pressure on conventional outflow facility. *Invest Ophthalmol Vis Sci*. 2008;49:275–281.
97. Lei Y, Overby DR, Boussommier-Calleja A, Stamer WD, Ethier CR. Outflow physiology of the mouse eye: pressure dependence and washout. *Invest Ophthalmol Vis Sci*. 2011;52:1865–1871.
98. Loewen RT, Roy P, Park DB, et al. A porcine anterior segment perfusion and transduction model with direct visualization of the trabecular meshwork. *Invest Ophthalmol Vis Sci*. 2016;57:1338–1344.
99. Peng M, Margetts TJ, Sugali CK, et al. An ex vivo model of human corneal rim perfusion organ culture. *Exp Eye Res*. 2022;214:108891.
100. Peng M, Margetts TJ, Sugali CK, et al. An ex vivo model of human corneal rim perfusion organ culture. *Exp Eye Res*. 2022;214:108891.
101. Karimi A, Razaghi R, Rahmati SM, et al. Modeling the biomechanics of the conventional aqueous outflow pathway microstructure in the human eye. *Comput Methods Programs Biomed*. 2022;221:106922.
102. Karimi A, Rahmati SM, Razaghi R, et al. Biomechanics of human trabecular meshwork in healthy and glaucoma eyes via dynamic Schlemm's canal pressurization. *Comput Methods Programs Biomed*. 2022;221:106921.
103. Karimi A, Khan S, Razaghi R, et al. Developing an experimental-computational workflow to study the biomechanics of the human conventional aqueous outflow pathway. *Acta Biomater*. 2023;164:346–362.
104. Vranka JA, Acott TS. Molecular differences in segmental regions of the trabecular meshwork. In: Knepper PA, Samples JR, eds. *Glaucoma Research and Clinical Advances 2016–2018*. Amsterdam: Kugler Publications; 2016:115–120.
105. Vranka JA, Bradley JM, Yang YF, Keller KE, Acott TS. Mapping molecular differences and extracellular matrix gene expression in segmental outflow pathways

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of the human ocular trabecular meshwork. *PLoS One*. 2015;10:e0122483.

106. Vranka JA, Staverosky JA, Reddy AP, et al. Biomechanical rigidity and quantitative proteomics analysis of segmental regions of the trabecular meshwork at physiologic and elevated pressures. *Invest Ophthalmol Vis Sci*. 2018;59:246–259.
107. Ethier CR, Chan D-H. Cationic ferritin changes outflow facility in human eyes whereas anionic ferritin does not. *Invest Ophthalmol Vis Sci*. 2001;42:1795–1802.
108. Vranka JA, Staverosky JA, Raghunathan V, Acott TS. Elevated pressure influences relative distribution of segmental regions of the trabecular meshwork. *Exp Eye Res*. 2020;190:107888.
109. Reina-Torres E, Baptiste TMG, Overby DR. Segmental outflow dynamics in the trabecular meshwork of living mice. *Exp Eye Res*. 2022;225:109285.
110. Strohmaier CA, McDonnell FS, Zhang X, et al. Differences in outflow facility between angiographically identified high- versus low-flow regions of the conventional outflow pathways in porcine eyes. *Invest Ophthalmol Vis Sci*. 2023;64:29.
111. Saraswathy S, Bogarin T, Barron E, et al. Segmental differences found in aqueous angiographic-determined high- and low-flow regions of human trabecular meshwork. *Exp Eye Res*. 2020;196:108064.
112. Bradley JMB, Kelley MJ, Zhu XH, Anderssohn AM, Alexander JP, Acott TS. Effects of mechanical stretching on trabecular matrix metalloproteinases. *Invest Ophthalmol Vis Sci*. 2001;42:1505–1513.
113. Abu-Hassan DW, Li X, Ryan EI, Acott TS, Kelley MJ. Induced pluripotent stem cells restore function in a human cell loss model of open-angle glaucoma. *Stem Cells*. 2015;33:751–761.

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