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Development and testing of a metabolic chamber for effluent collection during whole eye perfusions

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Abstract

Ocular hypertension is caused by dysregulated outflow resistance regulation by the conventional outflow (CO) pathway. The physiology of the CO pathway can be directly studied during ex vivo ocular perfusions. In addition to measuring outflow resistance generation by the CO tissues, perfusion media that is conditioned by CO pathway cells can be collected upon exiting the eye as effluent. Thus, contents of effluent include factors contributed by upstream cells that report on the (dys)functionality of the outflow tissues. Two methods have been used in the past to monitor effluent contents from perfused eyes, each with their limitations. To overcome these limitations, we designed and printed a metabolic chamber to accommodate eyes of different sizes during perfusions. To test this new chamber, human eyes were perfused for 4 hours at constant flow rate of 2.5µl/minute, while pressure was continuously monitored and effluent was collected every hour. Facility was 0.28 ± 0.16µl/min/mmHg for OD eyes and 0.33 ± 0.11µl/min/mmHg for OS eyes (n=3). Effluent samples were protein rich, with protein concentration ranging from 2700 to 10,000µg/ml for all eyes and timepoints (N=3). Effluent samples expressed proteins that were actively secreted by the TM and easily detectible including MYOC and MMP2. Taken together, our model provides a reliable method to collect effluent from *ex vivo* human eyes, while maintaining whole globe integrity.

7. Introduction:

Ocular hypertension is the primary risk factor for glaucoma (Flammer and Mozaffarieh 2007), a leading cause of blindness worldwide (Tham, Li et al. 2014). Importantly, ocular hypertension is also the only modifiable risk factor for glaucoma, caused by dysregulated removal of aqueous humor (AH) from the eye by the conventional outflow (CO) pathway. To leave the eye, AH passes through the components of the CO pathway in the following order: trabecular meshwork (TM), Schlemm's canal (SC), collector channels, intrascleral venous plexus and episcleral veins. While some resistance to AH outflow is generated in the distal portion of the CO pathway (McDonnell, Dismuke et al. 2018), the majority of outflow

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None

resistance is produced by the innermost region of the TM called the “juxtacanalicular (JCT)” region, where the inner wall of SC and the adjacent TM cells interact (Overby, Stamer et al. 2009, Tamm 2009).

Due to the avascularity of the CO tissues, the physiology of the CO pathway can be studied in isolation *ex vivo* by perfusing ocular preparations with mock AH (perfusate). In addition to measuring outflow resistance generation by the CO tissues, perfusate that is conditioned by CO pathway cells (effluent) can be collected as it exits the eye. Effluent contains many factors that can inform (dys)functionality of the outflow tissues. For example, TM cells secrete many autocrine and paracrine factors into passing AH/perfusate such as TGFB2, MMP2, MMP9, MYOC, ANGPTL7, NO, and VEGF (Alexander, Samples et al. 1991, Tripathi, Li et al. 1994, Fingert, Stone et al. 2002, De Groef, Van Hove et al. 2013, Hase, Kase et al. 2021, Reina-Torres, De Ieso et al. 2021, Praveen, Patel et al. 2022) that provide information about tissue homeostasis and health. Importantly, previous studies have demonstrated the utility of effluent isolation and analysis to inform ocular physiology/pathology (Table 1). For example, McDonnell et al. (2020) used effluent collected at various timepoints from human anterior segments transduced with an adenovirus engineered to have a shear stress-sensitive promoter (eNOS), driving a secreted reporter protein, secretory alkaline phosphatase (SEAP), to assess IOP-mediated effects on shear stress in the conventional outflow tract. In another study, Perruccio et al. (2008) used effluent collected from human anterior segments to monitor effects of dexamethasone on pigment epithelium derived factor secretion by conventional outflow cells. Comes and Borrás (2007) collected effluent from human anterior segments treated with glucocorticoid receptor (GR)-siRNA in the presence of dexamethasone to examine MYOC secretion by conventional outflow tissues. In a separate study, Perkumas et al. (2006) found myocilin associated with exosomes isolated from effluent collected from human anterior segments in organ culture. In an interesting study by Bahler et al. (2004), effluent was collected from human anterior segments with high initial pressures, and reinfused this effluent into the same eyes after pressure returned to baseline, resulting in a subsequent elevation of IOP. Lastly, Johnson et al. (1990) perfused whole globe human and bovine eyes with microspheres (0.18μm - l.1μm) and collected effluent to determine the percentage of microspheres of specific size passing through the human outflow system as a function of time, to inform filtration characteristics of the ocular outflow system.

In these studies effluent was either collected from the outside of anterior segments in organ culture clamped into perfusion chambers, (Johnson and Tschumper 1987) or isolated from perfused whole human eyes submerged in silicone oil (Johnson, Johnson et al. 1990). For the first method, anterior segments in organ culture are clamped into perfusion chambers, where the integrity of the whole globe is lost, and collection volumes from chamber are inconsistent because of the clamp ring, and a difficult dissection and custom perfusion chambers are required (Johnson and Tschumper 1987, Bahler, Fautsch et al. 2004, Comes and Borrás 2007, Perkumas, Hoffman et al. 2007, Perruccio, Rowlette et al. 2008, McDonnell, Perkumas et al. 2020). The second method was developed to solve some of these issues by collecting effluent from perfused whole human eyes submerged in silicone oil (Johnson, Johnson et al. 1990). The denser silicone oil caused the effluent to form bubbles as it exited the episcleral veins at the sclera, allowing the collection from the oil

surface using gentle suction from a Pasteur pipette. Since the viscosity of the oil is much higher than that of the effluent, the authors noted very little oil entered the Pasteur pipette. However, there are several limitations to this method. Firstly, the eyes are submerged in silicone oil, which is an unnatural environment and may not be conducive of normal outflow physiology. Moreover, there is a risk of silicone oil contamination in the effluent, whereby samples collected from the anterior chamber had to first be mixed with a little silicone oil to duplicate the conditions under which the perfusate was collected. Finally, there is risk of effluent loss when trying to separate the effluent from the oil. In response to limitations imposed by both methods, we designed and tested a new method to optimize collection of effluent from perfused whole eyes.

8. Materials and Supplies:

- Perfusate media (referred to as DBG):
  - 500mg anhydrous glucose (MP Biomedical, catalog number 152527)
  - DPBS, calcium, magnesium (Gibco™, catalog number 14040182)
- 60ml syringe (BD, catalog number 309653)
- 20ml syringe (BD, catalog number 302830)
- 10ml syringe (BD, catalog number 302995)
- Pressure Monitoring Tubing with Fixed Male Luer Lock and Female Fitting, Low Pressure, 36” (Medline, catalog number DYNJPMTBG36MF)
- Pressure Monitoring Tubing with Fixed Male Luer Lock and Female Fitting, Low Pressure, 24” (Medline, catalog number DYNJPMTBG24MF)
- Pressure Monitoring Tubing with Fixed Male Luer Lock and Female Fitting, Low Pressure, 12” (Medline, catalog number DYNJPMTBG12MF)
- Disposable Transfer Pipets (VWR®, catalog number 414004-018)
- Stopcock 4 Way (Smiths Medical, catalog number MX5341L)
- 25G x .75 in. BD Vacutainer® Safety-Lok™ Blood Collection Set with 12 in. tubing and luer adapter (BD, catalog number 367285)
- BD Luer-Lok™ Syringe (0.5ml) with attached needle (27G) (BD, catalog number 305620)
- 28 mm Diameter Syringe Filters, 0.2 µm Pore PES Membrane, Sterile, Individually Packaged (Corning®, catalog number 431229)
- We used the 202S Isotemp Digital Water Bath (Fisher Scientific, catalog number 15-462-2S). However, any temperature-controlled water bath with an adjustable lid (Fig. 1D) can be used.
- 2X Stamped Steel Two-Hole Support Stands with Rods (18 inch) (VWR®, catalog number 76293-250)

- 3X Cast Aluminum Clamp Holder (VWR®, catalog number 76293–416)
- 3X Clamp, three-prong extension, dual adjustment (VWR®, catalog number 80063-590)
- Legato 210 Two-Syringe Pump - Infuse/Withdrawal (WPI, catalog number SPLG210)

9. Detailed Methods:

9.1. Preparation:

1. The night before, prepare DBG by adding 1mg/ml of anhydrous glucose to DPBS (containing calcium and magnesium). Prepare 4×50mL tubes of the perfusate and warm to 37°C overnight to degas.
2. Enucleated human eyes (3 pairs) were obtained from Miracles in Sight/BioSight. For the purposes of this study, we perfused human eyes with death to perfusion time no greater than 24 hours. This time range was selected to be consistent with previous studies from short term perfusions of whole human eyes (Johnson, Johnson et al. 1990, Allingham, de Kater et al. 1992, Ethier and Chan 2001, Camras, Stamer et al. 2012, Ren, Li et al. 2016).
3. Place eyes on ice as soon as possible after death.
4. Set up perfusion apparatus as per Fig. 2 and Fig 3A, B, and C (set in **zero pressure sensor configuration**). NOTE: Fig. 3B is set to perfusion configuration). Syringe pump should be off. Check manifolds to clear any bubbles and leaks. If bubbles need to be cleared, ensure manifold is refilled with NaN_3. Fill all tubing and reservoirs with DBG as per Fig. 2 and avoid creating any bubbles. **NOTE: Push fluid slowly through tubing to prevent bubble formation. If bubbles form at junctions, detach luer-locked tubing, fill, and reattach with fluid-fluid contact**. Sherwood et al. (2016) provides a detailed summary as to the perfusion hardware and software set up.
5. The eye mounts are funnels on a stabilizing platform (Fig. 1A), with removable inserts to suspend the eyes (Fig. 1B). In collaboration with a local 3D printing company (Touchstone 3D, Cary, NC, USA), we designed and printed the eye mount (see supplementary files 1 and 2 for 3D blueprint). The eye mounts were printed on an SLA printer and were printed from a Formlabs material called Tough 2000. This material is not biocompatible, but it is possible to print our design using biocompatible materials. For the purposes of this new method, a biocompatible material is not necessary as the eye mount is solely used to support the eye and collect the effluent. The eye mounts are designed to accommodate eyes of different sizes that include human, bovine, rabbit and porcine. Fill water bath so that the legs of the eye mounts are submerged by 3cm of water (Fig. 1C, shaded area)
6. Place a probe-fitted thermometer inside the water bath chamber so that the probe is in the air and not submerged in the water. The water bath temperature was set

so that the humidified air temperature in the chamber is close to 37°C, to warm and prevent drying of the eyes. Keep checking the temperature throughout the experiment and adjust water bath temperature to keep the air temperature around 37°C.

9.2. Load calibration files and check pressure sensors:

7. Open perfusion software by double clicking the desktop icons which will open the W and B projects.
8. In each project, double click iPerfuse.
9. iPerfuse > Press the right facing arrow below ‘Edit’ in the top left corner to run the code.
10. iPerfuse > Setup > Basepath > User, enter your name in the text field. Hit the save button to the right to store this as default.
11. Perfuse > Setup > Basepath > Experiment, enter the name of your experiment. All data acquisition for this experiment will be saved in the same folder, separated by day of perfusion.
12. iPerfuse > Setup > Basepath > Press AutoName
13. iPerfuse > Setup > Pressure Sensor > Load
14. Calib_Liquid_Black/White.txt from folder origin (choose relevant one for software)
15. iPerfuse > Setup > Pressure Sensor > Save
16. iPerfuse > Setup > Pressure Sensor > Run. Pressure should be ~0.1
17. iPerfuse > Setup > Flow Sensor > Q Offset. Q-offset should be close to 0
18. Press “Run” to stop acquisition.

9.3. Zeroing pressure sensors to height of eyes:

19. Set manifolds to **perfusion configuration** and close off to pressure reservoir A at stopcock C (Fig. 2).
20. Fill empty containers with PBS and set in the eye mounts (to prevent wetting the mounts before use). NOTE: the packaging for the filters acts as an effective container here.
21. Use plyers to bend the perfusion needle at about halfway the length of the needle, at about a 30-degree angle to allow the needle to sit comfortably on the eye. The bevel should be facing up when cannulating (Fig. 3D).
22. Rest needles attached to the system in the PBS bath. You might need to use putty for extra support. Make sure PBS water level is about the same height as limbus of eye.
23. iPerfuse > Acquisition > Case > Eye

24. iPerfuse > Acquisition > Live

25. Check the pressure is zero. If it is not, adjust water level in the zero pressure reservoir until pressure is zero for black and white sides (Fig. 2).

26. Remove any PBS in eye mounts and dry.

27. Press “Live” to stop acquisition.

9.4. Cannulation:

NOTE: Refer to Fig. 2 and Supp. Video 1.

28. Set the eyes on the mounts with pupils facing up (Fig. 1C).

29. Place some putty on the edge of the support to use as a support for the needle. Try to keep putty dry.

30. iPerfuse > Acquisition > Live

31. Close flow from the syringe pump at the 4-way stopcock attached to flow sensor (stopcock B) but keep flow open to manifold and needle.

32. Open flow from each pressure reservoir A with stopcock C. Stopcock C should be open in all directions at this stage, so reservoir A is open to pressure and flow sensors.

33. Adjust water level (and if necessary, adjust height of the reservoirs with the clamps) of each pressure reservoir A until system pressure is as close to 10mmHg as possible for both white and black side.

34. Close flow from pressure reservoir A at stopcock C, and open flow to syringe pump at stopcock B. Stopcock B should be open in all directions at this stage. **NOTE: Refer to** Fig. 1 **and** 2

35. Remove any PBS in eye mounts and dry.

36. Use a 27G needle attached to a 0.5mL syringe to withdraw 200μL of aqueous humor (AH) from the anterior chamber of each eye. Be very careful not to insert the needle into the lens, and only draw the AH. Insert the needle through the center of the cornea. Gently withdraw AH from the anterior chamber, remove the needle, and place AH in a labelled tube. Store AH at −80C until/if needed for analysis.

37. Then recannulate the eyes with the bent 25G needle (Fig. 1E) attached to the perfusion setup (Fig. 2) as follows:

    a. Place some putty on the edge of the support to use as a support for the needle. Try to keep putty dry.

    b. Turn on syringe pump at 10 μL/min to ensure there is DBG flowing through the needle, and then cannulate into the posterior chamber (to prevent anterior chamber deepening) by inserting the needle through the same insertion point in the cornea as used for extracting AH. Use

tweezers to stabilize the eye. Set the needle tip between the iris and lens. **Be sure the bevel of the needle is facing upwards.**

c. Push the needle tubing (proximal to the needle butterfly) into the putty to hold it in place (Fig. 1E and D). Be careful not to displace the tip of the needle from beneath the iris.

d. Tape needle tubing to bath to prevent agitation of the needles when opening and closing the chamber lid.

e. Turn off syringe pump

f. Close flow from the syringe pump at the 4-way stopcock attached to flow sensor (stopcock B).

g. Open flow from each pressure reservoir A (10mmHg) at each stopcock C, which should be open in all directions at this stage. These reservoirs should now be open to pressure and flow sensors and should be set at 10mmHg. This step serves to reinflate the eyes to even pressures following the AH removal.

h. Check that the pressures of both eyes are similar are about 10mmHg and wait until flow rate has stabilized (approximately 10 minutes). If there are large fluctuations in flow rates, check for any bubbles in the needles, tubing, or manifolds. If the flow rates are too low (<1 μL/min), check if the needle is being pushed against the iris, thereby blocking flow. If the flow rates are too high (>8 μL/min), check for any leaks in the tubing, needle, or manifolds.

i. Close flow from pressure reservoir A at stopcock C, and open flow to syringe pump at stopcock B. Stopcock B should be open in all directions at this point.

9.5. Perfusion:

38. Set syringe pump to infuse at 2.5 μL/min and start infusion.

39. iPerfuse > Acquisition > Live (to stop Live)

40. iPerfuse > Acquisition > Record

41. To allow pressure to stabilize within the eye, perfuse eyes initially for 1 hour (see Supp. Fig. 1 for pressure dynamics over time), and then remove any effluent that has collected at the bottom of the funnel (Fig. 1A) and discard. This interval also allows for the effluent to pass through the episcleral veins to reach the surface of the eye, as it would likely be contaminated with blood (Sit, Gong et al. 1997).

42. The effluent exits via the episcleral veins and runs down the side of the eye (Fig. 1E) and pools at the tip of the funnel (Fig. 1A). During the perfusion, gently open the chamber lid and use a transfer pipette to remove effluent at 2H, 3H, and 4H mark by gently lowering the transfer pipette between the eye and the wall of the eye mount until the tip of the transfer pipette contacts the effluent. Put

effluent in labelled tubes and store at −80C until needed. For reference, see Supp. Video 2.

43. After the perfusion is complete, click iPerfuse > Acquisition > Record – to stop recording. Pressure was continuously monitored to calculate outflow facility (Fig. 4B)

9.6. End of Day

44. Remove needles from globes, dispose of in sharps

45. If fixation is required, make a small incision at the equator of each eye and submerge eyes in 4% PFA overnight at 4°C.

    NOTE: Change to PBS the day after and store at 4°C until ready to process tissue.

46. Empty all fixative into waste bottles and throw away tubing and plastics.

47. Drain eye baths and clean with 70% ethanol.

48. Rinse eye mounts with DI water and clean with 70% ethanol.

9.7. Effluent quality control:

A number of different types of proteins and molecules are secreted by the TM and deposited into the effluent, such as MMP-1, −2, −3, −9 (Samples, Alexander et al. 1993, Pang, Hellberg et al. 2003), TIMP-1 (Pang, Hellberg et al. 2003), MYOC (Tawara, Okada et al. 2000), connective tissue growth factor (Tomarev, Wistow et al. 2003) interleukin-6 (Liton, Luna et al. 2005), transforming growth factor-beta 1 (Tripathi, Li et al. 1993), prostaglandin E2 (Uchida, Shimizu et al. 2021, Uchida, Shimizu et al. 2021), and adenosine (Wu, Li et al. 2012). Furthermore, Yun et al. (1989) used immunofluorescence to show that the TM also secretes major constituents of the ECM including collagens (types I, III, IV, V and VI), laminin, fibronectin, and basement membrane-associated proteoglycans. So, to confirm fluid collected from the human eyes was effluent and not derived from condensation or another unintended source, we checked three parameters. Importantly, we used protein lysate from cultured TM cells treated with dexamethasone (not from perfused human eyes used in this experiment) only as a positive control (for MYOC) and negative control (for MMP) for the purposes of this experiment.

9.7.1. Protein concentration: To monitor protein content in AH effluent from the 3 pairs of human donor eyes, we performed a BCA assay (Pierce™ BCA Protein Assay Kit, catalog number 23227) (Fig. 4A). For AH, protein concentration ranged from 2500 to 7400μg/ml for all eyes. For effluent, protein concentration ranged from 2700 to 10,000μg/ml for all eyes and timepoints. N=3. For OD eyes, AH protein concentration (4841 ± 2340μg/ml) was not significantly different to effluent at 2H (6783 ± 3915μg/ml, p=0.59), 3H (6913 ± 2850μg/ml, p=0.47) or 4H (6523 ± 2329μg/ml, p=0.43). For OS eyes, AH protein concentration (4796 ± 2411μg/ml) was also not significantly different to effluent at 2H (7659 ± 4985μg/ml, p=0.52), 3H (7487 ± 4630μg/ml, p=0.52) or 4H (7621 ± 3765μg/ml, p=0.45). These data suggest that the effluent samples collected were protein rich.

9.7.2. MYOC expression: MYOC is secreted into the AH via several ocular tissues including ciliary body (Adam, Belmouden et al. 1997, Ortego, Escribano et al. 1997) and iris (Ortego, Escribano et al. 1997). MYOC is further secreted by the TM into effluent (Tawara, Okada et al. 2000, Fingert, Stone et al. 2002, Chowdhury, Madden et al. 2010). So, we checked to see if MYOC was present in our AH and effluent samples (Fig. 5A and C). To test this, we performed gel electrophoresis as previously described (De Ieso, Kuhn et al. 2022). AH and effluent samples were diluted to a protein concentration of 1mg/ml in 1X laemmli sample buffer with 100mM of dithiothreitol. Protein lysates were boiled for 5 min and 20μg of protein was loaded per well in 12% TGX Stain-Free polyacrylamide gels. Primary antibodies were diluted in 5% BSA in TBS-T, and blots were incubated in primary antibody solution at 4°C overnight, on a rocker. Rabbit polyclonal anti-MYOC primary antibody (homemade; 1/2500) was used to detect MYOC. We normalized to total protein using TGX Stain-Free™ FastCast™ Acrylamide Starter Kit, 12% (catalog number 1610184; Biorad). Dexamethasone-treated TM cell lysate (human TM136) was used as a positive control for MYOC. MYOC was prominent in the positive control, in the AH, and effluent from all samples and time points tested. The concentration of MYOC in the effluent samples was less than AH samples. We conclude this is due to the anterior chamber exchange of AH with protein-free DBG, which limited the contribution of MYOC in the effluent to conventional outflow cells/tissues.

9.7.3. MMP2 expression: The TM secretes MMP2 into the AH as it flows through the conventional outflow pathway and exits as effluent (Samples, Alexander et al. 1993), so MMP2 should also be expressed in the effluent samples. Therefore, we performed gel electrophoresis (as described above) to monitor MMP2 levels in the AH and effluent (Fig. 5B and C). Dexamethasone-treated TM cell lysate (human TM136) was used as a negative control for MMP2 as MMP2 is a secreted protein and its expression in cell lysate is downregulated by dexamethasone (Mohd Nasir, Agarwal et al. 2020). We normalized to total protein (as described above). Rabbit monoclonal anti-MMP2 primary antibody (catalog number 87809s; 1/1000; Cell Signaling Technology) was used to detect MMP2. As expected, MMP2 expression was absent in the dexamethasone-treated TM cell lysate. Presence of MMP2 was not detected in AH from 1 pair of human eyes and was detected at low levels in the other 2 pairs of eyes. MMP2 was abundant in all effluent samples from different donor pairs and timepoints, likely deposited by the TM and other conventional outflow pathway cells.

Thus, we showed that the effluent samples were protein rich, and expressed proteins that are known to be actively secreted by the TM including MYOC and MMP2 in perfused eyes.

We measured facility of all eyes to confirm outflow tissues were viable for the length of the experiment (Fig. 4B). Facility was 0.28 ± 0.16μl/min/mmHg for OD eyes and 0.33 ± 0.11μl/min/mmHg for OS eyes (n=3). These facility values are in the normal range for older human eyes, and were similar to previously published ranges for human eyes (Erickson-Lamy, Schroeder et al. 1990, Erickson-Lamy, Rohen et al. 1991, Acott, Kelley et al. 2014, Cha, Xu et al. 2016, Kazemi, McLaren et al. 2018, McDonnell, Dismuke et al. 2018). Thus, these data show that the eyes remained physiologically viable for the length of the experiment. We used eyes with death to perfusion time no greater than 24 hours,

and outflow facility within a normal range (0.2– 0.5 μl/min/mmHg) as an endpoint indicator for viable tissue. An additional quality control step may include tissue morphology by light and/or electron microscopy.

9.8. Statistics:

We used paired t-tests to determine statistical significance. Data is presented as mean ± SD. Significance defined as $p<0.05$. N=3 for all datasets.

10. Potential Pitfalls and Troubleshooting:

Taken together, our model provides a reliable method to collect effluent from *ex vivo* human eyes without the use of silicone oil, while maintaining whole globe integrity.

10.1. The advantages of this model include:

10.1.1. Simplicity of experimental setup and effluent collection: The experimental setup for this novel method is quick and straightforward. For the anterior segment organ culture model, the human whole globe donor eyes must first be carefully dissected, and the anterior segment must then be clamped onto the mount underneath a clamping ring that must create a watertight seal, while maintaining even pressure applied to the sclera. These extra steps are time consuming and introduce a higher risk for error. Our model does not require any prior dissection, and the whole eye can be set in the eye mount and remain stable without the use of clamps or gauze. There is also minimal risk for leakage, which can potentially occur if the clamp ring is not set properly in the anterior segment organ culture model. Furthermore, the silicone oil whole globe model presents higher risk for effluent contamination with oil, and higher risk for effluent volume loss in the collection process. It is also technically challenging to collect effluent mixed in silicone oil. For our model, risk of effluent contamination is low, and the effluent collection method is easy and maximizes the amount of effluent that can be collected as the effluent samples are isolated and restricted to the small space within the funnel tip.

10.1.2. Minimal mechanical stress on outflow tissues: In the anterior segment organ culture model, dissection of the tissue involves separation of the anterior segment and removal of lens, iris, and any leftover pigment. While there are advantages to this method, there is an increased risk for tissue damage or stress during the dissection and clamping process. This is particularly relevant since dissection and clamping of eye pairs must be very similar for interpretable results. Without the need to perform any prior dissection (except for the removal of connective tissue for non-human eyes) or mechanical clamping of the tissue to the mount, our new model for effluent collection presents a method with reduced risks for tissue damage or stress when preparing the eyes for the perfusion.

10.1.3. Maintenance of whole globe integrity and physiological environment: The anterior segment organ culture model does not allow for maintenance of whole globe integrity, with loss of structures such as the lens, iris, and posterior segment. Maintaining whole globe integrity is important to reduce stress on the conventional outflow tissues and to maintain an experimental environment similar to *in vivo* conditions. Moreover,

while the silicone oil whole globe model allows for maintenance of whole globe integrity, it does not facilitate an experimental environment that mimics physiological conditions as the globes are submerged in non-physiological silicone oil. Our model allows for maintenance of whole globe integrity in addition to maintenance of a physiologically similar environment to *in vivo* conditions. The humidified and temperature-controlled chamber keeps the eyes moist and warm as they would be *in vivo*, without having to submerge the eyes in oil.

10.1.4. Adaptability: Our novel method can be easily adapted to accommodate constant pressure perfusions instead of constant flow, for example, if a researcher wished to investigate the change in expression of a particular protein in the effluent following high and low IOP conditions. Furthermore, by performing dual cannulation, anterior chamber exchanges can be performed to perfuse drug, fluorescent microbeads, or fixative. In addition to human, the eye mounts are also compatible with bovine, rabbit, and porcine eyes. Our method has been used in a recent publication, where Kelly et al. (2023) performed dual cannulation for drug exchange, perfusing the anterior chamber of one pig eye with L-NAME and the contralateral eye with control perfusate. The perfusion was performed at constant pressure for 3 hours following a one-hour acclimation. In this study, Kelly et al. analyzed the effluent and found significantly lower nitrite levels in the L-NAME treated eye over time, demonstrating the utility and adaptability of our model and emphasizing that the cells are alive and responsive to drugs such as L-NAME. Thus, the adaptability of our new model ensures broad utility for the system, and for information that can be obtained from the effluent.

10.2. The limitations of this model include:

10.2.1. Shorter experimental duration: An advantage of the anterior segment organ culture model is that the highly vascular tissues are removed. The majority of the remaining tissue is avascular, such as the TM and cornea, so the duration of a given experiment can be longer as the avascular tissues receive sufficient sustenance from the perfusate to stay viable. The disadvantage of any whole globe perfusion protocol is that the experimental duration lasts over hours rather than days, as the vascular tissues such as the ciliary body and iris break down over time. Since changes in protein expression, secretion, or activation in response to a treatment can take longer than 4 hours, this method is limited to changes that can occur within a 4-hour timeframe.

10.2.2. Loss of protein: We showed that effluent collected with our model contained high amounts of total protein and we were also able to detect significant amounts of secreted proteins from the outflow pathway. However, as effluent is collected in a funnel after it runs down the side of the eye, there is a risk of protein loss due non-specific sticking to the ocular surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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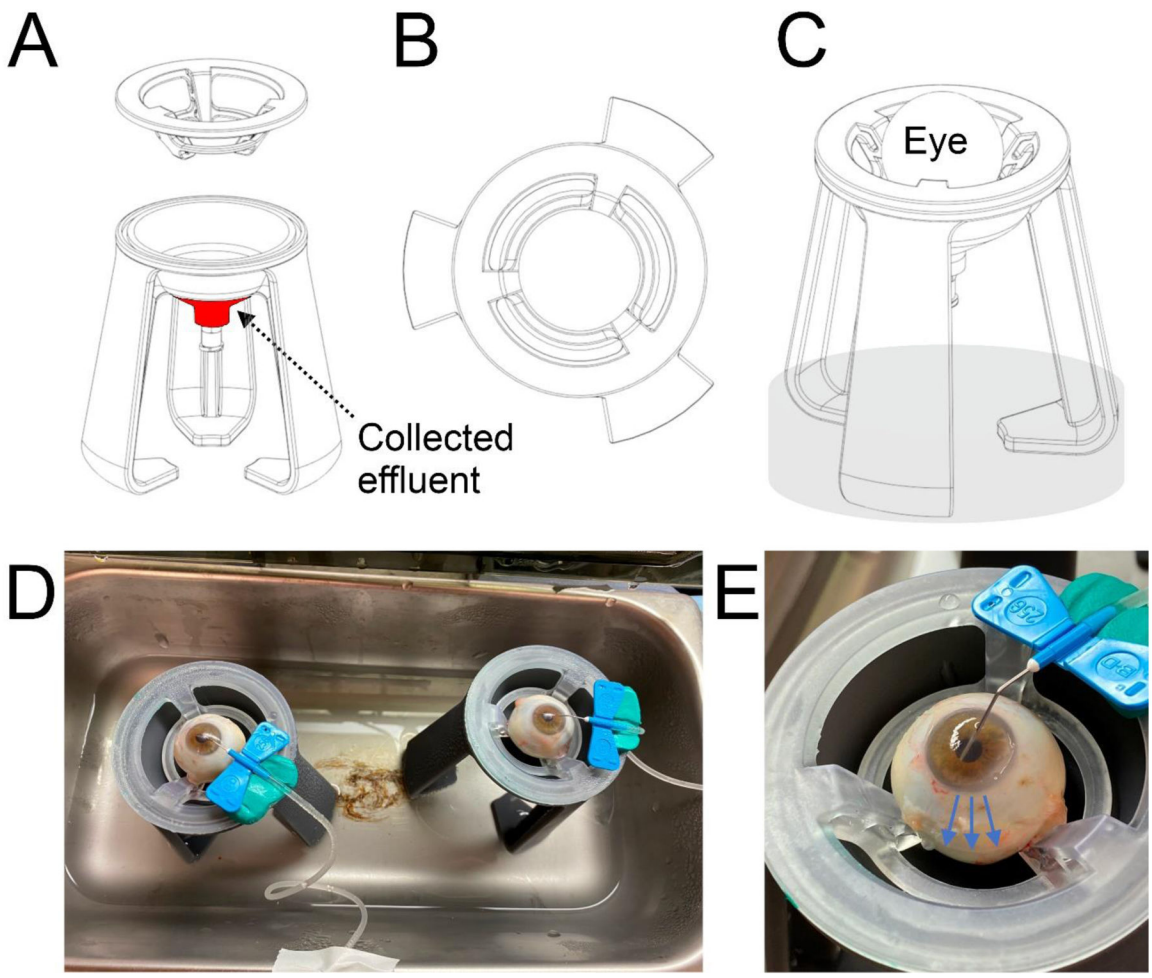

Figure 1: Design drawings for eye mount and images of metabolic chamber with human eyes.

(A) Side view of stand with removable insert to suspend the eye. Effluent collects at the tip of the funnel (red). (B) Top view of insert used to suspend the eye. (C) Side view of fully assembled eye mount with eye mounted, and water level in chamber is represented in transparent gray. (D) Humidified and temperature-controlled water bath (closed during perfusion) with eyes cannulated and set up on eye mounts. (B) Close up view of cannulated human eye set on removable insert. Effluent runs down surface of eye and collects at funnel tip (blue arrows).

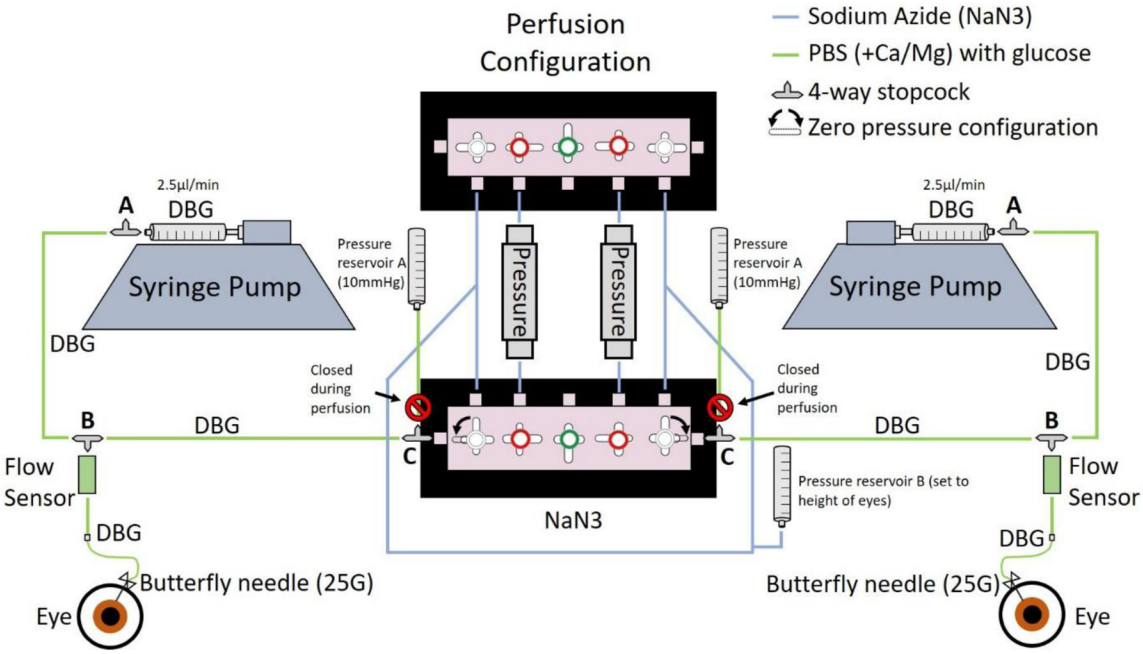

Figure 2: Schematic depicting perfusion setup.

Schematic depicting the perfusion setup. The syringe pump is set to constant flow (2.5µl/min), and each pressure reservoir A (closed to eye during perfusion) is set to 10mmHg (supported by stand and clamps as listed in materials) to re-inflate the eye following aqueous humor evacuation. Pressure reservoir 'B' is connected to both the black and the white side of the perfusions system. This reservoir is for zeroing the pressure at the height of the eyes prior to starting the perfusion. Phosphate buffered saline with calcium and magnesium (DBG) is used as perfusate. Sodium azide (NaN3) is a preservative reagent and is used to fill tubing that is not contributing to flow during the perfusion. A 25G butterfly needle is used for cannulation.

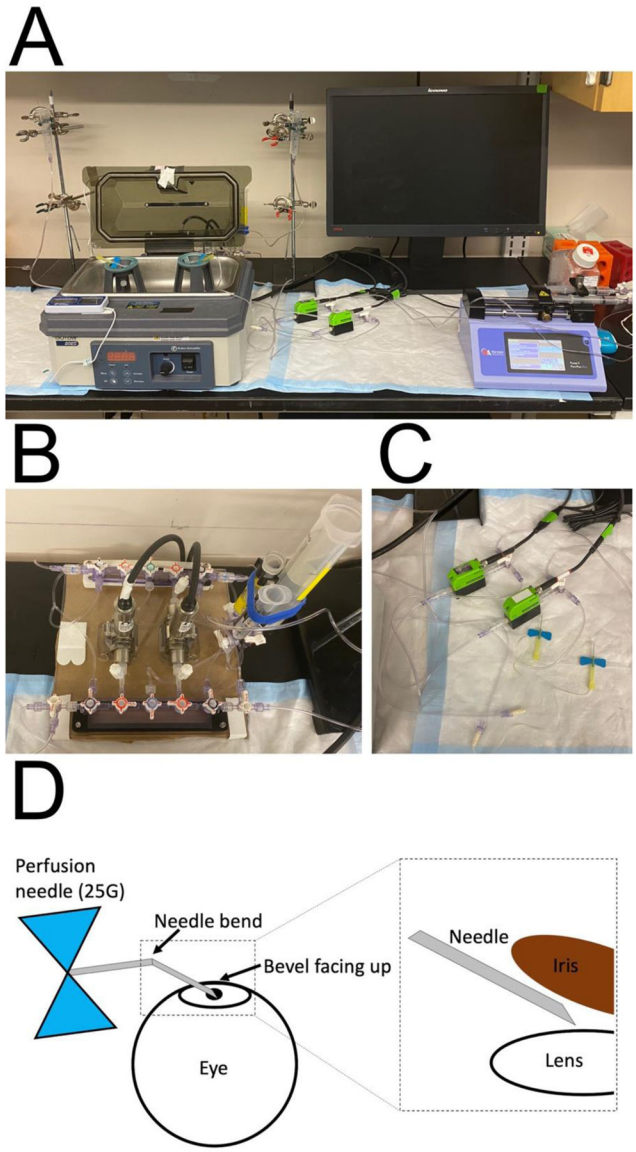

Figure 3: Images showing system setup and diagram depicting needle placement.

(A) Image showing entire experimental setup. Eye mounts are housed in humidification chamber, tubing is arranged and connected to manifold, flow sensor, reservoirs, and perfusion needles, according to Figure 2. Syringe pump set up is also shown. (B) An image of the manifold setup in perfusion configuration. (C) An image showing the tubing setup connected to the flow sensors. (D) A schematic demonstrating the configuration of the needle prior to cannulation. The needle is bent in the middle at approximately 30°, with bevel facing up. (Inset) Following cannulation, the needle is placed in the posterior chamber, between the iris and the lens, with the bevel facing upward toward the iris.

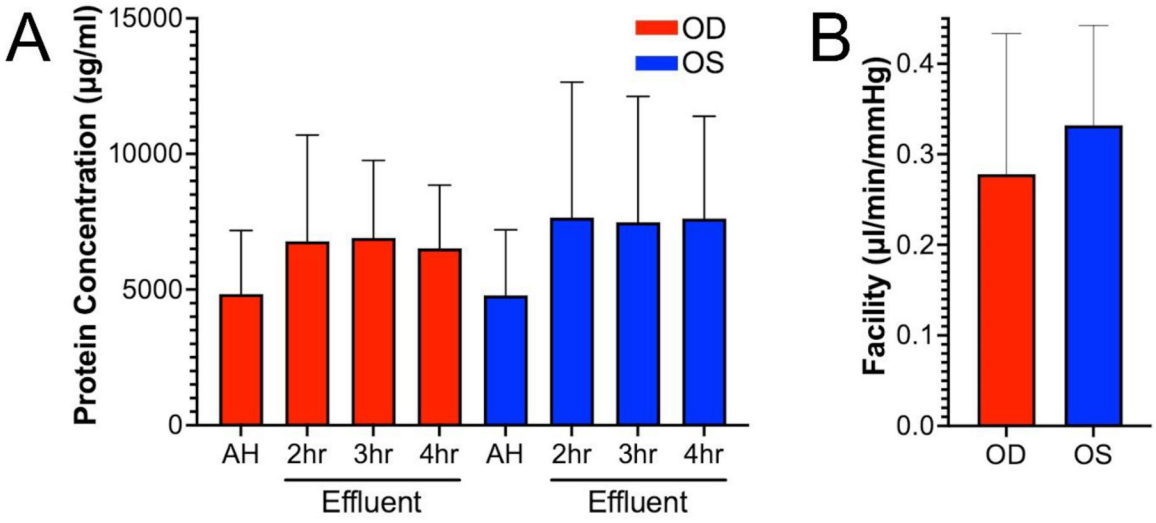

Figure 4: Effluent during whole eye perfusions contains abundant levels of protein.

(A) Protein concentration in the effluent samples collected from 3 pairs of human donor eyes was determined. For AH, protein concentration ranged from 2500 to 7400µg/ml for all eyes. For effluent, protein concentration ranged from 2700 to 10,000µg/ml for all eyes and timepoints. N=3. (B) Facility was calculated by averaging the pressure readings for each eye over a 3-hour period (between 1 and 4hrs). Then we used the constant flow rate (2.5µl/min) to calculate average facility over that time-period. Facilities were 0.28±0.16 and 0.33±0.11µl/minute/mmHg for OD and OS, respectively (mean±SD, n=3).

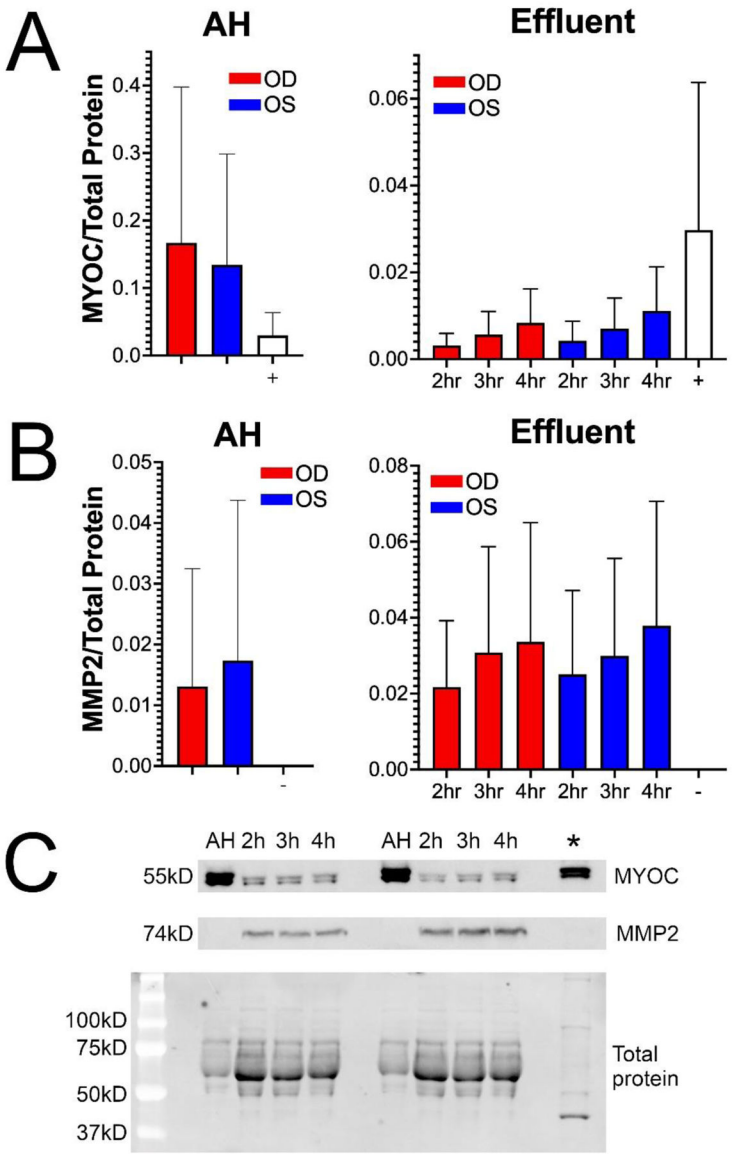

Figure 5: MYOC and MMP2 proteins are actively secreted by conventional outflow cells into effluent during whole eye perfusions.

(A) MYOC protein was present in all samples and timepoints as determined by western blot (n=3). As a positive control for MYOC (+), lysates were prepared from dexamethasone-treated cultured human TM cells. (B) MMP2 protein was abundant in effluent at all time points, and trace amounts of MMP2 was present in AH. There was no detectable MMP2 in the dexamethasone-treated cultured human TM cell lysate (n=3). Dexamethasone-treated cultured human TM cell lysate was used as a negative control for MMP2 (−). NOTE: Dexamethasone-treated cultured human TM cell lysate was not from perfused eyes in this experiment. (C) Representative blots from A and B. Total protein analysis was used for normalization.

Table 1:

Summary of previous studies monitoring contents in effluent from perfused eyes

Study	Measured in effluent	Method	Advantages	Disadvantages
(McDonnell, Perkumas et al. 2020)	Secretory alkaline phosphatase and nitrite	Human anterior segment organ culture perfusion	• Full control of perfusate contents • Removal of vascular tissues allows for longer perfusions	• Loss of whole globe integrity • Loss of effluent in clamp rings • Difficult dissection
(Perruccio, Rowlette et al. 2008)	Pigment epithelium derived factor			
(Comes and Borrás 2007)	Myocilin			
(Perkumas, Hoffman et al. 2007)	Myocilin-associated exosomes.			• Requires custom perfusion chambers
(Bahler, Fautsch et al. 2004)	Collected effluent from human anterior segments with high initial pressure.			
(Spiga and Borrás 2010)	Matrix metalloproteinase-1			
(Comes, Buie et al. 2011)	Myocilin and fibronectin			
(Johnson, Johnson et al. 1990)	Microspheres (0.18μm - l.1μm)	Perfusion of whole human eyes submerged in silicone oil	• Maintenance of whole globe integrity • No evaporation	• Physiologically unnatural environment • Risk of sample contamination with silicone oil • Loss of effluent when separating from silicone oil
(Sit, Gong et al. 1997)	Total protein			