Feedback-controlled constant-pressure anterior chamber perfusion in live mice

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Purpose: To describe live mouse, anterior chamber constant-pressure perfusion by an approach using feedback-controlled coupling of pressure and flow to maintain a preset pressure.

Methods: We established a microperfusion system that maintains a constant preset pressure in the anterior chamber of live mice by automatically regulating the microsyringe pump flow rate with a computer-controlled voltage feedback loop. Perfusion was by single-needle cannulation. We characterized the following in C57BL/6 mice aged 3–4 months in vivo: (i) pressure stability, (ii) pressure and flow rate reproducibility, (iii) total outflow facility, and (iv) anterior segment histology after perfusion.

Results: Twenty live mice underwent perfusion. Constant pressure was quickly attained and stably maintained. The coefficient of pressure variation over time during perfusion at a preset pressure was <0.001. The average coefficient of variation for repeat pressure and flow rate measurements was 0.0005 and 0.127, respectively. The relationship between flow rate and pressure was linear for perfusions between 15 and 35 mmHg. The total outflow facility was 0.0066 µl/min/mmHg. Perfusion system resistance (0.5 mmHg/min/µl) was negligible relative to the ocular outflow resistance (147 mmHg/min/µl) at physiologically relevant perfusion pressures of 15–35 mmHg. No histological disruption of the drainage tissue was seen following perfusion.

Conclusions: Predetermined pressure was stably maintained during constant-pressure perfusion of live mouse eyes by a method using feedback-controlled coupling of pressure and flow along with single-needle anterior chamber cannulation. Perfusion measurements were reproducible. This approach is potentially useful for exploring aqueous drainage tissue biology, physiology, and pharmacology in live mice.

Mouse and primate aqueous outflow systems share similar morphology and physiology [1-10], and many engineered mouse strains are available, making the mouse a promising model for studying aqueous outflow dynamics relevant to human biology and glaucoma. Anterior chamber microperfusion in live mice to measure aqueous outflow dynamics by different approaches has been reported [1-4]. The mouse eye is an order of magnitude smaller than the primate eye, with mouse anterior chamber volume about 5 µl compared with 135 µl in rhesus and 250 µl in humans [5-7]. Perfusing the anterior chamber of live mice is thus expected to be technically challenging. Not surprisingly, recent efforts to measure outflow dynamics have focused on enucleated eyes instead of live mouse eyes [8,9].

We have established apparatus and approaches for constant pressure anterior chamber perfusion in live mice. Given the small cornea and anterior chamber space of mice, it would seem preferable if perfusion studies were conducted by one-needle cannulation [8,9] instead of two-needle cannulation techniques often used in larger primate eyes [11,12] and adapted for mice [1,3]. We conducted constant-pressure perfusion by one-needle cannulation using a microsyringe pump electronically coupled to a pressure transducer and feedback control system. The feedback control system tracked transduced pressure to automatically vary pump perfusion flow rate to maintain pressure at a predetermined level. This methodology was applied to live C57BL/6 mice in which pressure stability and reproducibility, outflow facility, and perfusion system resistance were characterized.

METHODS

Animal husbandry and anesthesia: Mouse experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research. Approval was obtained from the University of Southern California Institutional Animal Care and Use Committee (IACUC). C57BL/6 mice, aged 3–4 months, were purchased from Charles River Laboratories (Wilmington, MA). The mice were raised and housed.
in air-filtered clear cages with a bedding of pine shavings, subjected to a 12 h:12 h light-dark cycle, and fed ad libitum. All perfusion measurements were performed between 12 PM and 5 PM.

Mice were anesthetized with a mixture of ketamine (60–85 mg/kg, Ketaject; Phoenix Pharmaceutical, Inc., St. Joseph, MO), xylazine (6–8.5 mg/kg, AnaSed; Lloyd Laboratories, Shenandoah, IA), and acepromazine (1.5–2.5 mg/kg; Boehringer Ingelheim, St. Joseph, MO) injected intraperitoneally. Anesthesia was titrated to achieve a depth facilitating stable anterior chamber cannulation and perfusion. One drop of topical proparacaine hydrochloride ophthalmic solution (0.5%; Akorn, Inc., Buffalo Grove, IL) was applied to the cornea before needle cannulation. Mice were placed on a warming platform or under a heating blanket to maintain body temperature during experiments.

**Perfusion apparatus:** A 5-mm-long 35-gauge needle (127 μm internal diameter; Medicom, Lachine, Quebec, Canada) attached to a micromanipulator (Type MM33 Rechts; Marzhauser, Wetzlar, Germany) and connected to perfusion apparatus was used to cannulate the mouse anterior chamber under microscopic guidance, as shown in Figure 1. The needle was connected to a rigid, noncompliant, Lectrocath tube (1 mm internal diameter, 15 cm length; Vygon Corporation, Montgomeryville, PA) attached to a three-way stopcock with a male nonvented cap (Edwards Lifesciences, Irvine, CA). The stopcock was connected on one hub to a flow-through pressure transducer (P75; Hugo Sachs, March–Hugstetten, Germany) and on a second hub to a calibrated glass microsyringe (50 μl, Hamilton 1705TLL; Hamilton Inc., Reno, NV) driven by a microsyringe pump (PhD Ultra; Harvard Apparatus, Holliston, MA). The pump was manufacturer rated to deliver a flow range of 0.1 nl/h to 220 ml/min with an accuracy within 0.35% and reproducibility within 0.05% (PhD Ultra datasheet). The pump was connected to a bridge amplifier (Octal Bridge Amp; AD Instruments, Colorado Springs, CO) that was connected to a digital input/output (I/O) data recording, display, and analysis hardware system (Powerlab; AD Instruments) integrated with LabChart 7.2 software (AD Instruments) on a computer.

There were further connections between an analog voltage pump feedback controller (STH Pump Controller; AD Instruments) and the microsyringe pump and digital I/O interface, and a split cable connection between the voltage pump controller and pressure transducer–amplifier linkup. The pump had been modified to receive a separate feedback connection from the pump controller, functioning as a real-time feedback control system coupling transduced pressure and perfusion flow. This permitted automatic variation of the pump flow rate to maintain a predetermined pressure level as set on the pump controller. Pressure and flow rate were displayed in Labchart software. The transduced pressure in the apparatus was calibrated using hydrostatic columns connected in series with the pressure transducer.

The perfusion apparatus was filled and primed with Dulbecco’s PBS [8,13,14] (DPBS; Mediatech, Corning) and calibrated before each experiment. The mouse anterior chamber was cannulated obliquely through the cornea and roughly parallel to the iris with a single new needle under microscopic guidance, taking care to avoid the iris and lens.

![Figure 1. Schematic diagram of experimental setup for feedback-coupled, single-needle, constant-pressure, anterior chamber perfusion in live mice. A 35-G needle for anterior chamber cannulation was connected via a rigid tube to a pressure transducer then to a microsyringe pump, bridge amplifier, and digital input/output (I/O) unit integrated with analytic software on a computer. The pump controller provided a feedback loop between the pressure transducer and the pump and amplifier.](image-url)
The needle insertion site on the cornea was monitored for leakage as judged by: (i) external pooling of leaking aqueous humor; (ii) alteration of fluorescein dye applied to the cannulation site in which clear fluid appeared with the leakage; or (iii) disruption of a silicone grease smear across the external corneal needle insertion site.

The pressure transducer was zero-referenced before anterior chamber cannulation. After anterior chamber cannulation, pressure was transduced until a stable baseline was reached. Pressure and flow rate were sampled 1,000 times per second, and recordings were displayed in real time on a computer monitor. The pump controller worked by continuously tracking the transduced pressure, comparing it with a predetermined set-point pressure value. An integrator analyzed the difference between set-point pressure and transduced pressure and used this data to adjust the microsyringe pump-slowing it down if transduced pressure increased, speeding it up if transduced pressure decreased [15].

For constant-pressure perfusion, the anterior chamber was perfused to achieve a stable constant pressure for at least 3 min during which time flow and pressure were recorded. This process was repeated for constant-pressure perfusions at physiologically relevant pressures of 15, 20, 25, 30, and 35 mmHg.

**Perfusion protocols and analysis:** To determine how stably the feedback-controlled system maintained pressure during constant-pressure perfusion, we analyzed the coefficient of variation (standard deviation [SD]/mean) of pressure over time for perfusion at different pressures. We then calculated the SD of pressure data captured every 10 ms during sequential 15-s blocks (1,500 measures per 15-s block) over 180 s. This provided information on how quickly and stably a new pressure set point was reestablished on presetting a new perfusion pressure.

To analyze reproducibility of constant-pressure perfusion, perfusion was performed at 25 mmHg and alternated with 15 mmHg to obtain repeat measurements. Perfusion at each pressure was conducted for 3 min and repeated five times for each animal. The pump had infusion and withdrawal modes that allowed perfusion pressure to be alternated between 15 and 25 mmHg; the withdrawal mode allowed pressure to be decreased from 25 to 15 mmHg. The coefficient of variation representing reproducibility was calculated from the repeat pressure and flow rate measurements.

To determine the total outflow facility of each animal, pressure and flow rate data were extracted from the software at a rate of one per 10 ms for 15,000 consecutive data points for each pressure condition. Data from the first 30 s (3,000 data points) were taken to occur during system stabilization and excluded. For the flow rate, data points were collected as voltage and converted to flow rate (µl/min) based on prior pump calibration. Perfusion flow rate represented the physiologic outflow rate, and perfusion pressure represented intraocular pressure (IOP). The relationship between pressure and flow rate for each animal was analyzed in scatter plots and modeled by regression analysis.

Total outflow facility was determined in live C57BL/6 mice as the slope of the flow rate (F; µl/min) versus pressure (P; mmHg) function. Flow versus pressure functions were plotted for each animal and modeled by regression analysis in a pressure range of 15–35 mmHg. Statistical analysis was performed in Excel® 2008 for Mac (Microsoft, Redmond, WA), StatPlusMac 5.7.5 (AnalySoft Inc., Alexandria, VA), and Minitab® 9.2 for Windows (Minitab Inc, State College, PA). Outflow resistance (R; mmHg/min/µl) was calculated as the inverse of the outflow facility [6,7].

Resistance of the perfusion system that included a 35-G needle was characterized. The tip of the 35-G needle connected to the perfusion apparatus was placed flat in a dish containing DPBS, with the tip submerged just beneath the fluid surface. DPBS was perfused through the needle into the dish by constant-flow perfusion at different flow rates, with the pressure at different flow rates recorded. The slope of the perfusion pressure versus flow rate function gave needle resistance.

To test the accuracy of pressure transduction through a 35-G needle, a second pressure transducer (PT#2) was added. Instead of cannulating the anterior chamber, the 35-G needle tip was sealed in the lumen of a closed hub of a rigid three-way connector directly attached to the second pressure transducer (PT#2). In this way the cannulated space, representing the anterior chamber, was transduced directly without an intervening 35-G needle to exclude possible confounding due to the needle. Thus, PT#1 and PT#2 provided separate measurements on each side of the 35-G needle of perfusion pressure and true intracameral pressure respectively, in our one-needle perfusion setup. Perfusion was conducted three times, each at different constant flow rates of 0, 0.3, 0.5, 1.7, and 2.9 µl/min.

**Histology:** Enucleated eyes were quickly embedded in Tissue-Tek Optimum Cutting Temperature compound for frozen sections. Sections were stained with hematoxylin and eosin (H&E). To determine drainage tissue integrity after perfusion, histological features after perfusion at pressures of 15-35 mmHg and in unperfused mouse eyes were examined by light microscopy.
Seventeen live C57BL/6 mice underwent constant-pressure anterior chamber perfusion within a physiologically relevant pressure range of 15–35 mmHg. Average body weight for the mice was 26.98 g. A further three animals were perfused to determine reproducibility at 25 mmHg, the mid-point between 15 and 35 mmHg. This was based on five repeat measurements at 25 mmHg for perfusion, alternating between 15 mmHg and 25 mmHg.

**Pressure stability:** Stable pressure was achieved during perfusion at all constant pressures, as shown in representative perfusion pressure and corresponding flow rate traces in Figure 2 for perfusion at pressures between 15 and 55 mmHg. Once steady-state pressure was achieved, perfusion was conducted at that pressure for at least 3 min. Figure 2 shows that constant pressure was reached quickly and maintained over time at different perfusion pressures. Flow rate increased gradually with perfusion pressure; flow rate at 15 mmHg was 0.086±0.015 µl/min (mean±standard error of the mean [SEM], n = 17) and at 35 mmHg was 0.21±0.03 µl/min. The pressure coefficient of variation over time during constant-pressure perfusion was below 0.001 for all pressure conditions, and this could be maintained over longer periods as needed (data not shown).

**Pressure and flow rate reproducibility:** Pressure and flow rate traces for constant-pressure perfusion at alternating pressures of 15 and 25 mmHg are shown in Figure 3A. Flow traces corresponding to descending pressure (oblique downward pressure trace) occurred when the pump and pump controller were switched to manual control fluid withdrawal in constant-flow mode, during which fluid was withdrawn from the eye. This was performed to decrease pressure from 25 to 15 mmHg to permit the 15/25 mmHg alternating pressure protocol. Measurements recorded during the fluid withdrawal period were not used for analysis. Negative flow rate values
corresponded to a zero flow rate right after reinitiating feedback control but before pressure equilibration (preconstant nonequilibrated pressure phase); these recordings were not used either. Only recordings captured during feedback-controlled constant-pressure perfusion were used for analysis. Average coefficient of variation for the repeat pressure and flow rate measurements was 0.0005 and 0.127, respectively (n = 3 animals).

Flow–pressure relationship: Flow rate increased with increasing perfusion pressure, as shown in Figure 2. The relationship between mean perfusion flow rate and pressure fit a linear function within a physiologically relevant pressure range of 15–35 mmHg ($y = 0.0066x - 0.034; R^2 = 0.97; n = 17$), as shown in Figure 3B. Flow rate variance (SEM) was generally low, although it increased for constant-pressure perfusions at higher pressures.

System and needle resistance: Figure 3C shows that transduced pressure due to combined perfusion system and needle resistance rose linearly to 0.6 mmHg at 1.4 µl/min, a flow rate far exceeding flow rates for mouse perfusion at 35 mmHg (0.2 µl/min), the upper limit of physiologically relevant perfusion pressure. Our model indicated that at a flow rate of 0.2 µl/min, resistance accounted for 0.1 mmHg of the 35 mmHg of pressure transduced at this flow rate. Perfusion system resistance was 0.5 mmHg/min/µl, 312 times less than the outflow resistance of 3–4-month-old C57BL/6 mice (147 mmHg/min/µl). Total resistance in the perfusion apparatus and 35-G needle was thus considered negligible.

Figure 3. Total outflow facility and system resistance. A: Pressure and flow rate traces during alternating constant-pressure measurements at 15 and 25 mmHg. X–X’ (withdraw; upper case for pressure, lower case for flow), X’–Y (preequilibration, preconstant pressure), and Y–Y’ (feedback controlled constant-pressure perfusion) indicate different phases of alternating pressure and flow traces. B: Pressure versus flow rate relationship for 3–4-month-old mice (n = 17). A linear relationship is seen ($y = 0.0066x - 0.034; R^2 = 0.97$). C: Pressure–flow rate regression functions for the perfusion system with a 35-G needle (n = 4) compared with C57BL/6 anterior chamber perfusion using the same needle (n = 17). Slope of the regression function represented resistance (mmHg/min/µl). ■ needle; ● mice eyes; error bars, standard error of the mean.
and not expected to induce significant artifacts in pressure transduction.

**Drainage tissue morphology following perfusion:** Figure 4 shows representative histology of the anterior chamber and aqueous drainage tissue of unperfused eyes (A) and eyes perfused at pressures between 15 and 35 mmHg (B). No morphological differences in the angle structures, trabecular meshwork, and Schlemm’s canal that represented tissue disruption were seen between perfused and unperfused eyes.

**Validation of pressure measurements:** Figure 5 shows the setup for testing the accuracy of the transduced perfusion pressure during one-needle mouse eye perfusion. At flow rates of 0.30±0.012 (mean ± SD) and 0.52±0.0002 µl/min (Figure 5), the pressure difference between PT#1 and PT#2 (PT#1–PT#2) was 0.012±0.0086 and 0.13±0.029 mmHg, respectively, with the difference increasing with flow rate according to a linear model of \( y = 0.48x - 0.11 \), where \( y = PT#1–PT#2 \) and \( x = \) flow rate. This difference reflected the miniscule error in transduced pressure due to needle resistance as depicted in Figure 3C. Thus, at a physiologic outflow rate of 0.086 µl/min (at 15 mmHg), transduced pressure (PT#1) marginally overestimated true IOP (represented by PT#2) by a mean of 0.059 mmHg or 0.02%. At a perfusion flow rate of 0.5 µl/min (5.5X the physiologic flow rate), transduced pressure (PT#1) overestimated IOP (PT#2) by a mean of 0.13 mmHg. This marginal difference was considered negligible.

**Figure 4.** Representative histology of the angle structures and outflow tract of C57BL/6 eyes after perfusion. A: Unperfused eye; B: eye perfused in a pressure range of 15–35 mmHg. Eyes were snap frozen immediately after the perfusion experiments. Perfused eyes were histologically similar to unperfused control eyes. Scale bar, 50 µm.

**Figure 5.** Validation of pressure transduction accuracy. A pressure transducer (PT#1) was connected to a 35-G needle that was inserted into the lumen of a closed hub of a rigid three-way connector directly attached to a second pressure transducer (PT#2). At flow rates of 0.30±0.012 (mean ± standard deviation [SD]) and 0.52±0.0002 µl/min, the pressure difference between PT#1 and PT#2 was 0.012±0.0086 and 0.13±0.029 mmHg, respectively. This represented a small artifactual increase in transduced pressure in PT#1 due to needle resistance. Thus, at an outflow rate of 0.086 µl/min corresponding to physiologic mouse intraocular pressure (IOP) of 15 mmHg, PT#1 transduced pressure marginally overestimated IOP (PT#2) by a mean of 0.059 mmHg or 0.02%. This marginal difference was considered negligible.
DISCUSSION

We have described a method for perfusing live mouse eyes using a feedback-controlled constant-pressure perfusion approach. This microperfusion system combined with single-needle cannulation provided stable and reproducible measurements during constant-pressure perfusion at different pressures. The flow–pressure relationship was linear in a physiologically relevant pressure range of 15–35 mmHg.

We modified a classical experimental setup, aiming to provide simplification and ease of use with perfusion of the mouse anterior chamber [11,12,16]. A microsyringe pump rated to accurately deliver small volumes (nano to microliter range) commensurate with perfusing the miniscule mouse anterior chamber was used. In our setup, the pump was electronically coupled to a pressure transducer and feedback control system to automatically vary flow rate to sustain a predetermined pressure, permitting constant-pressure perfusion. Hence, instead of invariably applying a constant pressure to the anterior chamber, anterior chamber pressure was held constant by varying the flow rate to maintain a preset pressure based on real-time pressure transduction. A variation of this concept has been applied to successfully perfuse postmortem enucleated mouse eyes [8,9]. Resistance in our perfusion system that included a 35-G needle was negligible relative to the C57BL/6 outflow resistance and perfusion flow rates that we used. Our one-needle cannulation used standardized, commercially available 35-G needles and was simple to set up and well suited to the miniscule mouse anterior chamber. In our one-needle perfusion, transduced pressure only marginally overestimated intracameral pressure by 0.02% at physiologic IOP.

We quantified the moment-to-moment variation in perfusion pressure to determine pressure constancy. Typically, steady-state constant-pressure recordings were reached quickly, well within a minute of a change in set-point pressure, mirroring a similar constant-pressure perfusion method used in enucleated C57BL/6 eyes [8]. Pressure stability was reflected in pressure coefficients of variation of under 0.001 over time during constant-pressure perfusions. Low flow rate variance, especially at lower perfusion pressures, was reflected in narrow error bars (SEM) in flow–pressure functions. We noticed incremental widening of flow rate variance at higher perfusion pressures, however, possibly reflecting drainage tissue instability with higher pressure. Reproducibility is important as it defines the extent to which fluid dynamic changes can be detected with confidence. Pressure stability permits equilibrium to be reached quickly, allowing efficient experiments (shorter experiments, fewer animals). We believe our method fairs well on both counts. To the best of our knowledge, prior publications on mouse perfusion have not included stability and reproducibility information. Our data fill this gap and provide a basis for future reporting and meaningful comparisons between different perfusion approaches.

All components of our perfusion system were rigid and noncompliant. The pressure trace square wave shift and quick flow rate equilibration right after changing the set-point pressure reflected this low compliance, agreeing with data from constant-pressure perfusion in enucleated C57BL/6 eyes [8]. This resulted in efficiencies; typical constant-pressure perfusions between 15 and 35 mmHg could be completed in under 30 min per animal.

We validated pressure transduction in our system by determining how closely transduction through a 35-G needle represented true intracameral pressure. This was tested using the same setup with a pressure transducer (PT#1; Figure 5), as described for live animal perfusion. Instead of cannulating the cornea, however, the 35-G needle was inserted into the lumen of a closed hub of a rigid three-way connector directly attached to a second pressure transducer (PT#2). In this way, the cannulated space (representing the anterior chamber) was transduced without a second intervening needle, which would have confounded determination of putative transduction artifacts due to the first needle. A small differential between PT#1 and PT#2, amounting to 0.059 mmHg (0.02%), was seen at physiologic flow rates, with this differential increasing linearly to 0.13 mmHg at a flow rate 5.5X that of the physiologic flow rate. We considered this miniscule differential to be negligible.

Total outflow facility measurement by constant-pressure perfusion was 0.0066 µl/min/mmHg for our 3–4-month-old mice. Our outflow facility measurements were close to previously reported values: 0.005 µl/min/mmHg for C57BL/6 in vivo, 0.0066 µl/min/mmHg for C57BL/6 ex vivo, and other live mouse strains, such as National Institutes of Health Swiss white and Balb/c mice (range 0.005 to 0.018 µl/min/mmHg) using different techniques [1-3,8,14]. We are now planning to study the effect of perfused drugs on outflow facility. To facilitate this, we are developing and validating techniques for anterior chamber fluid exchange by single-needle cannulation to allow for accurate drug dosing in the live mouse anterior chamber.

Comprehensive characterization of other aqueous dynamic parameters has been reported [1-3] and is beyond the scope of our study, which solely aimed to characterize pressure stability and reproducibility during constant-pressure perfusion using a feedback control system in live mice. We have characterized this approach in C57BL/6 mice,
a principal background strain for engineered mice, providing a rational basis for exploring questions pertinent to glaucoma and its therapy in mouse models.

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