

Noninvasive Assessment of Aqueous Humor Turnover in the Mouse Eye

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PURPOSE. To develop a noninvasive test for monitoring changes in aqueous humor turnover in the mouse eye.

METHODS. After topical instillation of fluorescein, the rate of decay of fluorescence from aqueous humor and cornea was monitored in Black Swiss, C57 Bl6, and DBA 2J mice with a microscope equipped with epifluorescence and a charge-coupled device (CCD) camera.

RESULTS. The rate of decay of fluorescence was identical in right and left eyes over an approximately 70-minute measurement period. The rate of decay was similar in normal mice aged 2 and 18 months. Pilocarpine and latanoprost, known to enhance aqueous humor outflow in humans, accelerated the decay of fluorescence. Levobunolol, known to inhibit aqueous humor inflow in humans, slowed decay. Dimethylamiloride, an inhibitor of the Na⁺,H⁺ exchanger that is known to act on cultured cells of both the ciliary epithelium and trabecular meshwork and to lower mouse intraocular pressure (IOP), enhanced decay. DBA 2J mice, in which secondary glaucoma develops, displayed a slower decay of fluorescence at 18 months of age than age-matched unaffected animals.

CONCLUSIONS. Monitoring decay of fluorescence provides a noninvasive index of aqueous humor dynamics in the mouse eye that facilitates study of ocular hypotensive drugs and mouse models of glaucoma. Coupled with knowledge of IOP, it permits semiquantitative conclusions about the relative roles of aqueous humor inflow and outflow in conditions with altered IOP. Based on this approach, dimethylamiloride appears to lower mouse IOP primarily by enhancing outflow of aqueous humor. (*Invest Ophthalmol Vis Sci.* 2003;44:722-727) DOI: 10.1167/iovs.02-0386

The mouse is an advantageous nonprimate mammal for investigating the genetic control of physiologic function¹ and the pharmacology of intraocular pressure (IOP).² By electron microscopy, the outflow tissues of the mouse show morphologic similarities to those of the primate, and it has been suggested that they serve a comparable function.³ Mouse IOP also responds in parallel fashion to human IOP after administration of drugs known either to inhibit aqueous humor inflow or to stimulate aqueous humor outflow.² We have adapted the

servonull micropipette system to monitor mouse IOP reliably over periods as long as 45 minutes.² With this technique, novel approaches to lowering IOP have been tested in wild-type^{4,5} and transgenic mice.⁶

Despite the central importance of IOP,⁷⁻⁹ measurements of pressure alone do not distinguish between changes caused by primary alterations in aqueous humor inflow or outflow. Validated invasive and noninvasive methods can provide this complementary information in eyes of many experimental animals and humans, but application of many of these techniques to the mouse is limited by the small size of the eye. Adaptation of fluorescent tracer techniques to the mouse eye would be particularly advantageous because the approach is noninvasive and has little effect on aqueous dynamics in other species.¹⁰ Since the initial demonstration by Ehrlich¹¹ in which systemically injected fluorescein appeared in the aqueous humor, fluorescent tracer techniques have been modified by many investigators, especially by Goldmann^{12,13} and Jones and Maurice,¹⁴ to develop a quantitative method.^{10,15}

In the current study, we here adapted the established method of fluorescein clearance^{10,14} to the mouse eye to learn whether this approach could be useful in assessing the relative contributions of changes in aqueous humor inflow and outflow to changes in IOP. We validated the fluorescein clearance method by comparing right and left eyes, measuring the effects of ocular hypotensive drugs known to enhance outflow (pilocarpine, latanoprost) and reduce inflow (levobunolol) in humans and other mammals, and taking measurements in the DBA 2J mouse, in which pigmentary glaucoma develops and serves as a model of human glaucoma. Finally, we demonstrated that the fluorescein clearance approach can identify the major site of action of a novel ocular hypotensive drug, the Na⁺,H⁺-exchange inhibitor dimethylamiloride.

MATERIALS AND METHODS

Animals

Three strains of mice were studied. Black Swiss outbred mice of mixed sex, 7 to 9 weeks old and approximately 30 g in weight, were purchased from Taconic Farm (Germantown, NY). DBA 2J mice, in which anatomic changes in the anterior chamber angle and glaucoma with increased IOP are known to develop,^{1,16} were obtained from Jackson Laboratories (Bar Harbor, ME) and studied at 18 months, when weighing approximately 25 g. As the control for the DBA 2J animals, age-matched C57 Bl6 mice¹⁷ of mixed sex and weighing approximately 30 g were also purchased from Jackson Laboratories. Animals were housed under a 12-hour light-dark illumination cycle, with light onset at 7:00 AM, and were provided unrestricted access to food and water. All measurements were performed with animals under general anesthesia induced by intraperitoneal ketamine (100 mg/kg) and xylazine (9 mg/kg). Xylazine was specifically included to prolong the period of measurement. All procedures conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Fluorescein Loading

Using the method described herein, autofluorescence of the cornea, aqueous humor, and iris was measured before fluorescein was added

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and those data were subtracted from all subsequent measurements. The correction constituted less than 10% of the total measurement. A droplet of fluorescein in saline (10 μ L, 0.02%, pH 7.8, 304 mOsm) was applied topically to both eyes. After 5 minutes to permit ocular absorption of fluorescein, excess fluid was absorbed with filter paper. Eyes and lashes were carefully irrigated with saline solution (600 μ L), and excess fluid was absorbed by filter paper. Because drugs penetrate the mouse cornea rapidly,² the next fluorescence reading was taken immediately after excess fluid was absorbed and was used as the peak fluorescence (100%, $t = 0$) for normalizing all subsequent measurements of decay of the signal.

Drug Applications

After the reference determination of maximum fluorescence at $t = 0$, a 10- μ L droplet of pilocarpine (1%; Alcon, Fort Worth, TX), latanoprost (0.005%; Xalatan, Pharmacia & Upjohn, Kalamazoo, MI), levobunolol (0.5% Betagan; Allergan, Hormigueros, Puerto Rico), or dimethylamiloride (1 mM; Sigma, St. Louis, MO) was applied to the experimental eye (randomly right or left) of drug-treated mice. The contralateral control eye received 10 μ L of vehicle, comprising isotonic saline solution (310 mOsm) with 0.003% benzalkonium chloride (Sigma), which is typically added to ophthalmic solutions to enhance penetration of topical drugs.¹⁸ Dimethyl sulfoxide (0.5%; DMSO) was included in the dimethylamiloride solution to facilitate solubilizing the drug and was also included in the control vehicle administered to the contralateral eyes of that series. Vehicle solution containing 2% to 8% DMSO and 0.03% benzalkonium itself has no effect on IOP in the mouse.^{2,4} In comparing right and left eyes and in studying DBA 2J mice, no drug was administered, and both eyes received vehicle alone. After 5 minutes of exposure either to vehicle or drug, the residual fluid was absorbed gently with filter paper ($t = 5$ minutes). Measurements for analysis of the disappearance of fluorescence from control and experimental eyes were initiated 15 minutes after instilling drug and at 10-minute intervals thereafter.

Fluorescence Measurements

Under general anesthesia, mice were placed prone on black paper to avoid reflection of extraneous light and positioned to be fully supported on the stage of an upright microscope (Eclipse 600; Nikon, Tokyo, Japan) equipped for epifluorescence with an FITC cube (emission filter: 480 nm/40 nm; dichroic mirror: 505 nm; barrier filter 530 nm/50 nm) and with a 10 \times objective (PlanFluor; Carl Zeiss, Thornwood, NY) with a 16-mm working distance. Mice were oriented with the sagittal plane of the head perpendicular to the microscope stage, without external fixation of the head. The stage was moved to position the objective over the superior region of the iris halfway between pupil and limbus, and the microscope was manually focused to a depth approximately halfway between cornea and iris before the fluorescein was loaded (Fig. 1A). The eyes of individual mice were studied alternately by translational displacement of the microscope stage, with refocusing as needed using the technique just described. We estimate the central anterior chamber depth of the mouse to be approximately 400 μ m, based on a plastic-embedded tissue section of a formalin-fixed eye (data not shown), and estimate the focal depth of the 10 \times objective to be approximately 11 μ m at the wavelengths pertinent to fluorescein. Although this objective captures light from out-of-focus regions, we found in initial experiments that fluorescence intensity varied by no more than 6% from that recorded with optimal positioning before the objective became so defocused as to fail to satisfy these positioning criteria (data not shown).

After the fluorescein was loaded, excitation was performed only every 10 minutes to minimize photobleaching. Images were obtained with a charge-coupled device (3-CCD) digital camera and stored for later analysis (Toshiba America, Irvine, CA). After capturing all images for an individual mouse, the stored images were analyzed on computer (Image Pro Plus; Media Cybernetics, Silver Spring, MD). Each image was converted into its red, green, and blue components, allowing

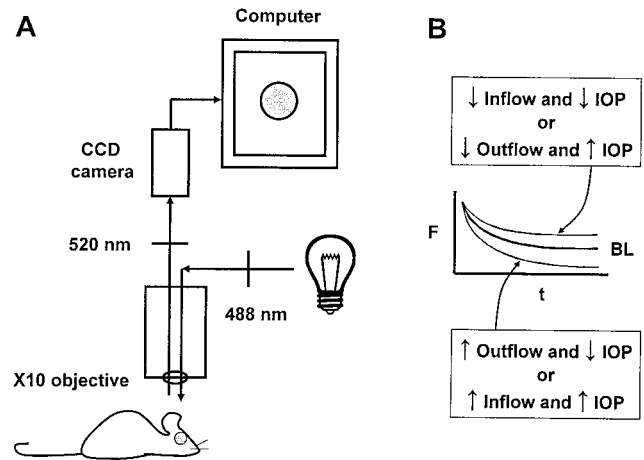


FIGURE 1. Schematic of method of measurement. (A) With a long-working-distance, 10 \times objective, fluorescein in the aqueous humor and cornea is excited at 488 nm and the emission at 520 nm is recorded with a CCD camera and computer. The emitted light is quantified from a 200 \times 200- μ m area of image (Fig. 2). (B) As illustrated, slowing of the baseline (BL) decay of fluorescence can reflect a reduction in either inflow or outflow and acceleration of decay can arise from an increase in either inflow or outflow. The possibilities can be distinguished by measuring IOP as well.

quantification of only the emitted green light's intensity. Data were obtained from a computer-outlined area of the image, 200 \times 200 μ m (Fig. 2). The fluorescence (F) at each time point was normalized (in percent) as $F = (\text{green intensity}) \times 100 / (\text{green intensity at } t = 0)$.

Statistical Analysis

Results are reported as the mean \pm SEM and n represents the number of experiments or eyes. The probability of the null hypothesis was estimated by two-way analysis of variance (ANOVA). For the particular series comparing the right and left eyes, the criteria of normality and equal variance were not fulfilled, and the ANOVA was applied to rank-transformed data.

RESULTS

Measurements in Normal Mice

To clarify the origin of the fluorescent signal, fluorescein was applied to the cornea for only 1 minute before washout in three experiments, and measurements were initiated 1 minute later. Figure 3 shows that the signal intensity steadily increased to a peak at 5 minutes and then decayed. These results led us to take the first measurement of fluorescence 5 minutes after application of fluorescein as the maximum signal, for purposes of normalization in all subsequent experiments.

The data in Figure 3 are qualitatively similar to experimental results obtained in human eyes, in which the larger eye size greatly facilitates measurements from individual ocular compartments.¹⁹ After application of fluorescein to the human eye, corneal fluorescence is high initially and then rapidly decays. In contrast, the anterior chamber fluorescence first rises rapidly and then begins to decline before reaching a phase characterized by the same slow, steady rate of decay in both the cornea and anterior chamber.¹⁹ The initial period is thought to represent diffusion of fluorescein from cornea into aqueous humor, whereas the later decline of both signals is due to the flow of tracer out of the eye. The observation that the late signal decays slowly from both aqueous humor and corneal compartments at the same rate suggests that the two compartments are at equilibrium. By analogy with the time course of fluorescence

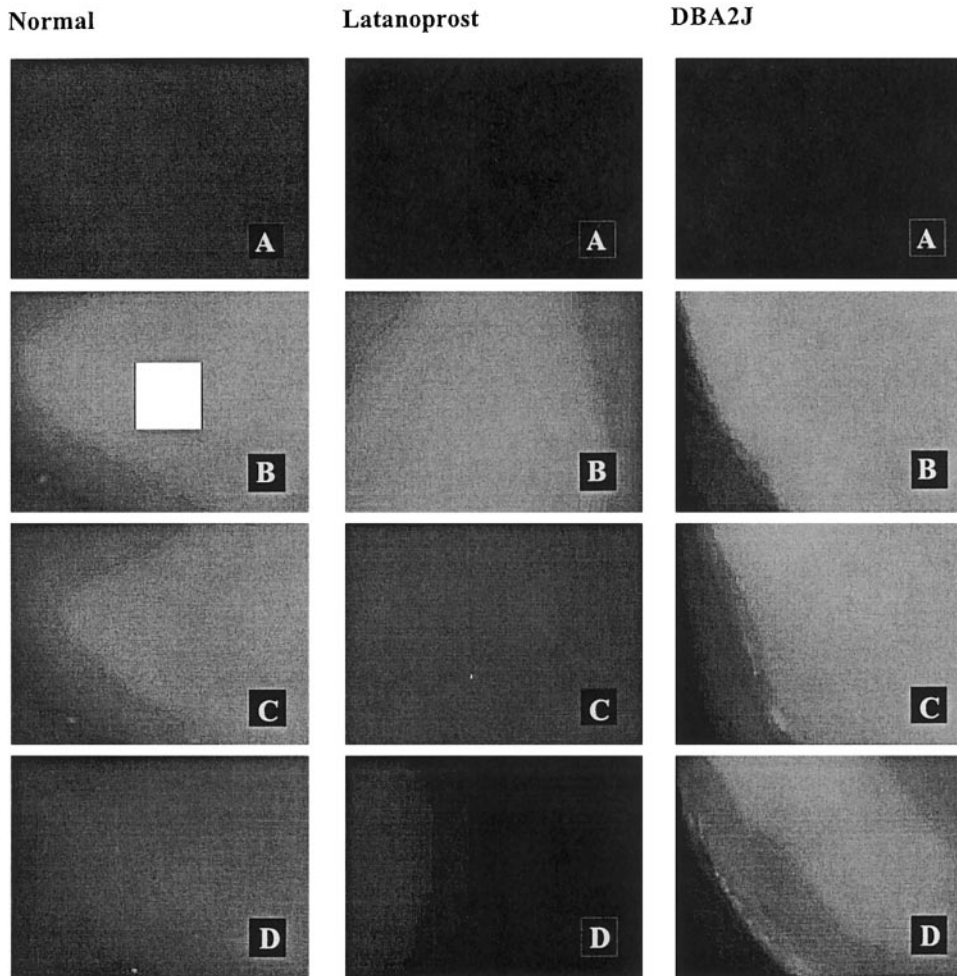


FIGURE 2. Time course of images of cornea and aqueous humor from a control mouse (*left*), latanoprost-treated Black Swiss outbred mouse (*middle*), and a DBA2J mouse (*right*) before and during the period of decay of fluorescence. The images in the four rows were obtained before application of fluorescein (A), directly after loading of fluorescein (B, peak fluorescence, $t = 0$), and at 25 (C) and 45 (D) minutes after loading of fluorescein. The *box* (B, Normal) identifies the position of the computer-outlined $200 \times 200\text{-}\mu\text{m}$ area in which the intensity of the emitted light was measured.

in the human eye, Figure 3 suggests that fluorescence in the aqueous humor of the mouse provides the predominant contribution to the total monitored signal.

The decay of fluorescence was measured over periods as long as 70 minutes, the maximum duration permitted by the anesthesia regimen. Qualitative disappearance of fluorescence from control and experimental eyes is readily apparent by

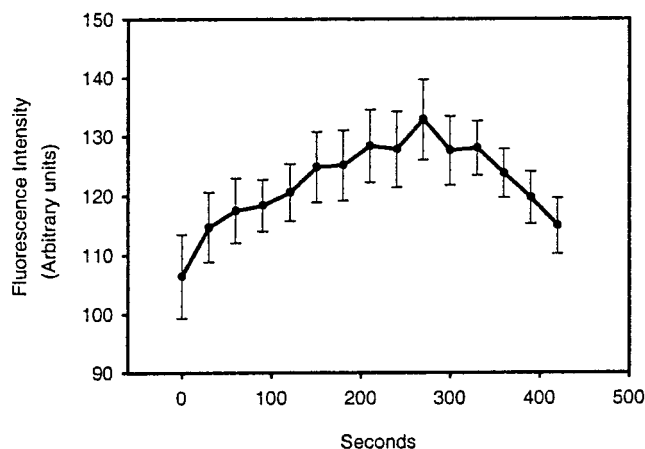


FIGURE 3. Early time course of fluorescence in aqueous humor and cornea in control Black Swiss mice ($n = 3$). The fluorescence intensity reached a maximum at approximately 5 minutes and thereafter decayed.

visual inspection of the raw images of Figure 2. The figure presents representative time courses from a normal mouse under baseline conditions (left column), from a drug-treated normal mouse displaying accelerated decay of fluorescence (middle column), and from a glaucomatous mouse displaying slowed decay (right column). As a test of reproducibility of the method, we found that the decay in the right and left eyes of seven normal Black Swiss mice was essentially equivalent and nearly identical with that in the vehicle-treated eyes in the separate set of mice used for drug testing (Fig. 4A, $P > 0.3$). This agreement between contralateral eyes provides a validation of the microscope alignment technique and supports the feasibility of adapting the fluorescein clearance technique to the small eye of the mouse.

Measurements in Glaucomatous Mice

The IOP of wild-type C57 Bl6 mice is similar to that of Black Swiss mice.⁶ Consistent with this similarity in IOP, the time courses of disappearance of fluorescence were similar in 18-month old C57 Bl6 and 7- to 9-week-old Black Swiss mice (Fig. 4B, $P = 0.81$). In contrast, 18-month old DBA 2J glaucomatous mice displayed a much slower disappearance of fluorescence than either the age-matched C57 Bl6 ($P < 0.001$) or the younger Black Swiss mice ($P < 0.001$; Figs. 2, 4B).

Responses to Drugs

Pilocarpine (Fig. 5A), which enhances aqueous humor outflow in humans and reduces IOP in both the human and the mouse,² accelerated disappearance of fluorescence in Black Swiss mice

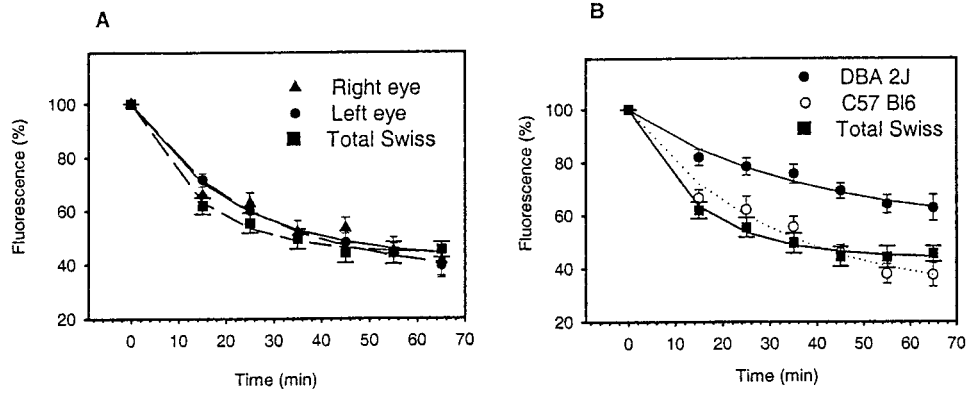


FIGURE 4. (A) Rate of decay of fluorescence in control Black Swiss outbred mice (Total Swiss, 7–9 weeks old, $n = 34$), and a comparison of right and left eyes in a separate series of experiments ($n = 7$). All data have been corrected for background autofluorescence, which accounted for less than 10% of the total fluorescence. In Figures 4 and 5, data points are the mean \pm SE joined for illustrative purposes by least-square monoexponential fits. (B) Comparison of the disappearance of fluorescence in control C57 Bl6 mice ($n = 8$) and DBA 2J glaucomatous mice ($n = 15$), both at 18 months of age. For ease of comparison, the total Black Swiss mouse data (A, Total Swiss) are also plotted in (B).

($P < 0.001$). Latanoprost (Figs. 2, 5B), which also increases aqueous humor outflow in the human and lowers IOP in the human and the mouse,² accelerated fluorescence decay ($P < 0.001$), as well. In contrast, levobunolol, known to decrease aqueous humor inflow and IOP in humans, slowed the rate of disappearance of fluorescence (Fig. 5C, $P < 0.001$).

Like pilocarpine, latanoprost, and levobunolol,² the Na^+, H^+ antiport inhibitor dimethylamiloride lowers mouse IOP.⁵ However, because of the novelty of the IOP result, it is unknown whether dimethylamiloride lowers IOP in any species primarily by reducing aqueous inflow through inhibition of ciliary epithelial cell transport^{20,21} or by enhancing aqueous

outflow, possibly by reducing trabecular cell volume.²² With the present methodology, dimethylamiloride enhanced the decay of fluorescence (Fig. 5D, $P < 0.001$).

DISCUSSION

The present work describes a simple, noninvasive adaptation of fluorescent-tracer techniques to characterize aqueous flow in the mouse eye. Brubaker^{10,15,23} has emphasized the analogy between these techniques and the measurement of renal clearances. Within that context, the present approach is analogous

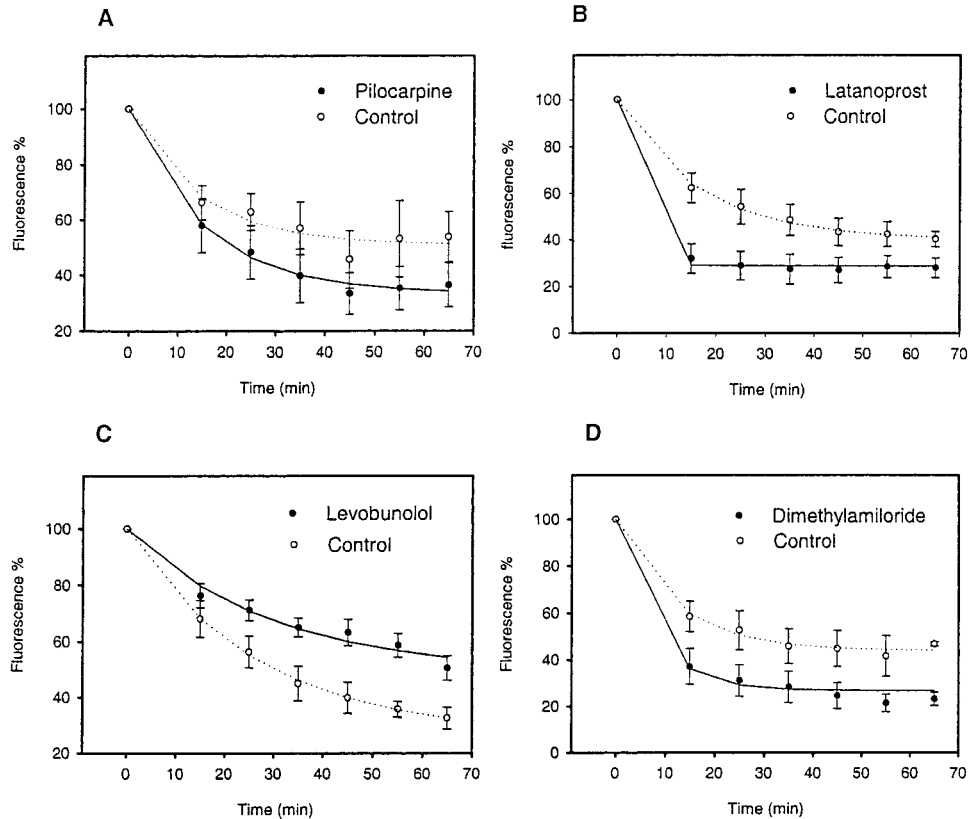


FIGURE 5. Effectiveness of pilocarpine (A, $n = 9$, $P = 0.003$), latanoprost (B, $n = 8$, $P < 0.001$), levobunolol (C, $n = 8$, $P < 0.001$), and dimethylamiloride (D, $n = 9$, $P < 0.001$), in altering the rate of disappearance of fluorescence in control Black Swiss mice. In each case, data are included from the contralateral vehicle-treated eyes.

to the application of clearance to measure fractional urinary excretion of a test solute. Measurement of fractional excretion does not permit quantification of the separate rates of tubular reabsorption and secretion in different segments of the nephron, but does identify the overall dominant renal effect as a net secretion into, or net reabsorption of solute from, the glomerular filtrate. Similarly, in the present context, monitoring the disappearance of fluorescence from the anterior segment does not quantify either inflow or outflow rates in absolute terms, but does permit assessment of whether an experimental perturbation of IOP is mediated through a dominant effect on the inflow or outflow of aqueous humor (Fig. 1B). Conversely, in models of glaucoma, measurement of disappearance of fluorescence can verify that IOP is elevated because of reduced outflow, although it cannot distinguish between the conventional pathway or uveoscleral pathway. Thus, measurements of IOP and fluorescein clearance are complementary in assessing the dynamics of aqueous humor.

In validating a fluorescein clearance approach for the mouse eye, we compared right and left eyes, compared different strains of normal mice, tested a mouse model of secondary glaucoma, and measured the responses to drugs with known mechanisms of action on human aqueous dynamics. Right and left eyes from a given strain displayed the same rate of disappearance of fluorescence (Fig. 4A), and the rate of decay was similar in control untreated eyes in two different strains of mice at different ages (Fig. 4B). In contrast, DBA/2J mice displayed the slowest rate of decay of the animal strains studied under control or experimental conditions (Fig. 4B). The DBA/2J mice have secondary glaucoma with essential iris atrophy, pigment-related disease in the trabecular meshwork, and elevated IOP.^{1,16} Coupled with the elevation of IOP, the slow fluorescein clearance establishes reduced aqueous humor outflow as the dominant functional abnormality. The responses to ocular hypotensive drugs (Fig. 5) were also consonant with the known mechanisms of drug action in humans and other mammals and followed the basic principles underlying the current approach (Appendix). Enhancing outflow (with pilocarpine or latanoprost) accelerated, and reducing inflow (with levobunolol) slowed, the disappearance of fluorescein.

To learn whether the fluorescein clearance approach could be useful in identifying the mechanism of drug action in mice, we studied the Na⁺,H⁺ antiporter inhibitor dimethylamiloride, which lowers mouse IOP.⁵ In vitro, dimethylamiloride inhibits transport by ciliary epithelial cells in intact tissue²¹ and in culture²⁰ and also reduces the volume of trabecular meshwork cells cultured from the outflow pathway. Given its ocular hypotensive action, dimethylamiloride's effect in acceleration of decay of fluorescence establishes that its dominant mechanism of action is to enhance aqueous humor outflow.

The current work thus provides a noninvasive method to study aqueous dynamics that can identify the dominant mechanisms underlying both elevation of IOP in glaucomatous mouse models and reductions of IOP produced by novel drugs. Together with a reliable method to measure IOP, use of the fluorescein clearance approach should facilitate application to glaucoma research of the molecular genetics and pharmacology now possible in the mouse.

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APPENDIX

Analysis of fluorescein washout from the anterior segment is potentially very complex. The time course can reflect lateral diffusion of fluorescein within the cornea to the limbal vessels,

release from and reuptake of fluorescein by the cornea, posterior movement of fluorescein, and changes in inflow, outflow, and volume of the anterior chamber.^{10,23} In addition, because of its high lipid solubility, fluorescein is not strictly limited to the extracellular space.

Analysis of the turnover of fluorescein is facilitated by making some simplifying assumptions, consonant with extensive studies in human subjects²³: (1) During the period when fluorescence disappears, exchange between cornea and anterior chamber is much faster than exit from the anterior chamber; (2) during the period of measured disappearance of fluorescence, inflow, outflow, and volumes of anterior chamber and corneal compartment are in a quasisteady state; and (3) the dominant basis for decay of the signal is convective loss of fluorescein through conventional (trabecular) and unconventional (uveoscleral) outflow pathways. In humans, 95% or more of topically loaded fluorescein leaves the eye through convective flow to the iridocorneal angle.²³ Under these assumptions, it can be shown that

$$dF/dt = -F \cdot J_{in} / (A \cdot \ln\{P_{epi} + [(J_{in} - J_{unc})/C]\} + V_{Cor}) \quad (A1)$$

where F is fluorescence measured from the sample, including contributions from aqueous humor and cornea; J_{in} is the inflow rate of aqueous humor; A is a constant incorporating a coefficient of stiffness (change in pressure divided by change in

volume); P_{epi} is the episcleral venous hydrostatic pressure; J_{unc} is outflow through the unconventional pathway; C is outflow facility; and V_{Cor} is volume of corneal compartment contributing to the measured fluorescence.

The qualitative effects of drug- or disease-induced changes in the time course can be predicted from equation A1 (Fig. 1B). Drugs that reduce IOP by reducing inflow slow the decay of fluorescence (decrease dF/dt) because the numerator (proportional to J_{in}) decreases more rapidly than the denominator (a logarithmic function of J_{in}). In contrast, drugs that lower IOP by enhancing conventional outflow facility decrease the denominator (a logarithmic function of $1/C$), thereby accelerating loss of the fluorescent signal. Similarly, drugs that lower IOP by enhancing unconventional outflow decrease the denominator (a logarithmic function of $-J_{unc}$), thereby increasing the rate of decay.

We also measured decay of fluorescence in a model of glaucoma with elevated IOP thought to arise from a reduction in outflow facility. The decrease in C led to an increase in the denominator in equation A1, thus slowing the decay. In principle, an increase in IOP could also arise from an experimentally induced elevation of aqueous humor inflow. In that case, the decay of fluorescence would be accelerated, because the numerator (proportional to J_{in}) increases more rapidly than the denominator (a logarithmic function of J_{in}).