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Elevated pressure influences relative distribution of segmental regions of the trabecular meshwork

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Abstract

Elevated intraocular pressure (IOP) is the primary risk factor for glaucoma and is the only treatable feature of the disease. There is a correlation between elevated pressure and homeostatic reductions in the aqueous humor outflow resistance via changes in the extracellular matrix of the trabecular meshwork. It is unclear how these extracellular matrix changes affect segmental patterns of aqueous humor outflow, nor do we understand their causal relationship. The goal of this study was to determine whether there are changes in the segmental outflow regions with perfusion in normal eyes, and whether these regions change during the IOP homeostatic response to elevated pressure. Using human anterior segment perfusion organ culture, we measured the amount of high flow (HF), intermediate flow (MF), and low flow (LF) regions before and after 7 days of perfusion at either physiologic pressure ("1x") or at elevated pressure ("2x"). We found a small but significant decrease in the amount of HF regions over 7 days perfusion at 1x pressure, and a twofold increase in the amount of MF regions over 7 days perfusion at 2x pressure. Small positional differences, or shifts in the specific location of HF, MF, or LF, occurred on a per eye basis and were not found to be statistically significant across biological replicates. Differences in the amount of segmental flow regions of contralateral eyes flowed at 1x pressure for 7 days were small and not statistically significant. These results demonstrate that perfusion at physiologic pressure had little effect on the distribution and amount of HF, MF and LF regions. However, the overall amount of MF regions is significantly increased in response to perfusion at elevated pressure during IOP homeostatic resistance adjustment. The amount of both HF and LF regions was decreased accordingly suggesting a coordinated response in the TM to elevated pressure.

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Keywords

trabecular meshwork; aqueous humor outflow; anterior segment; segmental outflow; glaucoma; elevated intraocular pressure

1. Introduction

Glaucoma is a leading cause of irreversible blindness worldwide (Quigley, 1996). Elevated intraocular pressure (IOP) is the main risk factor for glaucoma (Quigley, 2011). Normally intraocular pressure is maintained by generating resistance to aqueous humor outflow as it exits the eye (Acott and Kelley, 2008; Grant, 1951, 1963; Johnson, 2006). This process is regulated by the cells residing in the deepest layer of the trabecular meshwork (TM) or the juxtacanalicular TM region, and the adjoining inner wall endothelium of Schlemm's canal (SC). These regions constitute the conventional (trabecular) outflow pathway (Ethier, 2002; Johnson et al., 2002; Johnson et al., 1990; Overby et al., 2009; Tamm, 2009). In particular, the extracellular matrix (ECM) of the TM is believed to be an important component of the aqueous humor outflow resistance (Acott and Kelley, 2008; Bradley et al., 1998; Keller et al., 2009a; Keller et al., 2009b; Keller et al., 2007).

The cells of the TM and inner wall of SC reside in a dynamic environment in which they are responsive to constant changes in intraocular pressure (Johnstone and Grant, 1973; WuDunn, 2009). As part of their normal adaptive response, TM cells in the outflow pathway sense pressure changes as distortion or stretching and they respond by adjusting the outflow resistance to keep intraocular pressure (IOP) within acceptable limits (Acott, 2014; Acott et al., 2014; Stamer and Acott, 2012). This homeostatic process involves matrix metalloproteinase-initiated turnover of the ECM and biosynthesis of new ECM molecules, as well as changes in cytoskeleton, and cytokine secretion (Bradley et al., 2001; Keller et al., 2007; Vittal et al., 2005; WuDunn, 2009). Thus healthy TM cells are good biomechanosensors that function to regulate IOP accordingly. In glaucoma the TM tissue has been shown to be twenty-fold stiffer than normal age-matched TM (Last et al., 2011). This loss of compliance would presumably affect its ability to sense pressure changes and adapt. Importantly, we recently demonstrated that the homeostatic mechanism is indeed impaired in glaucomatous eyes (Raghunathan et al., 2018).

Aqueous outflow has long been recognized to be segmental, or non-uniform, around the circumference of the eye, though the mechanistic details are poorly understood. Many tracers have been used to visualize this variability in fluid outflow across the trabecular meshwork, and all reflect a similar pattern of non-uniformity (Braakman et al., 2015; Chang et al., 2014; de Kater et al., 1989; Ethier and Chan, 2001; Hann et al., 2005; Lu et al., 2008; Vranka et al., 2015). The relative levels of fluorescence in the trabecular meshwork of perfused anterior segments have previously been shown to be directly related to the amount of fluid flow within a corresponding region. (Chang et al., 2014; Keller et al., 2011) For example, high fluorescence within the TM indicates an active- or high-flowing region, whereas, low or no fluorescence indicates little to no fluid outflow. Our recent work has identified biomechanical differences between segmental outflow regions in normal eyes in

which low flow regions were found to be approximately two-fold stiffer than high flow regions (Vranka et al., 2018). We have also shown molecular differences at the RNA and protein level between the low and high flow regions (Vranka and Acott, 2017; Vranka et al., 2018). In comparing the relative amounts of low and high flowing regions we showed that glaucomatous eyes have relatively more low flow regions than normal eyes (Raghunathan et al., 2018). This suggests that the segmental flow regions are directly affected in the pathology of glaucoma. Cell numbers in the TM have been shown to decrease with age, and this difference is more pronounced in glaucomatous eyes (Alvarado et al., 1981). From our recent studies it is evident that dramatic differences in flow regions and loss of IOP homeostasis appear to be defining characteristics of glaucomatous eyes compared to normal eyes. In spite of these observations, it is not known how the relative amounts of the segmental regions of the TM and their distribution patterns change over time, or under the influence of elevated pressure. Therefore the goal of this study was to quantitate the outflow regions of the TM during perfusion to determine whether there are changes in normal eyes, and whether these regions change in response to elevated pressure.

2. Materials and methods

2.1. Anterior Segment Perfusion Culture

Anterior segment perfusion culture is an established technique to study outflow facility *ex vivo* (Johnson and Tschumper, 1987). Use of human donor eye tissue was approved by the Oregon Health & Science University Institutional Review Board and experiments were conducted in accordance with the tenets of the Declaration of Helsinki for the use of human tissue. Human eye tissue was obtained postmortem from Lions VisionGift, Portland, OR, USA, and we obtained no information that could lead to identification of a tissue donor. Length of time from death to stationary culture was less than 48 hours and anterior segments were initially placed into serum-free stationary organ culture for 5–7 days to facilitate postmortem recovery (Acott et al., 1988). The age range was 65 – 90 years old and the average age of the donors' eyes for all experiments in this study was 77.6 ± 3.0 years. Donor demographics are included in the Supplemental Demographics Table S1. After stationary culture, human anterior segments were perfused with serum-free Dulbecco's Modified Eagle's Medium (a 1:1 mixture of high and low glucose DMEM) containing 1% Penicillin/Streptomycin/Fungizone, at constant pressure (8.8 mmHg) with an average flow rate of 1–9 $\mu\text{l}/\text{min}$, which is similar to normal physiological rate and pressures (minus episcleral venous pressure) *in vivo* (Johnson and Tschumper, 1987). Anterior segments were perfused at a continuous pressure of 8.8 mmHg pressure (or "1x"), or were elevated to 17.6 mmHg (or "2x") perfusion pressure to produce a pressure challenge to trigger intraocular pressure homeostatic responses (Acott et al., 2014) as noted in the figure legends and as was described previously (Raghunathan et al., 2018; Vranka et al., 2015; Vranka et al., 2018). Perfusion flow rates were recorded for the duration of the perfusion time. The flow rates at 1x and 2x perfusion pressure were normalized and graphed for all donor eyes (shown in Figure 1). All anterior segments perfused at elevated pressure demonstrated a robust IOP homeostatic response over the full 7-day time period of perfusion.

2.2. Labeling During Perfusion

After baseline flow rates were stabilized (approximately 24 hours after starting perfusion), fluorescently-labeled amine-modified 200nm Fluospheres (red) (Thermo Fisher Scientific, Waltham, MA, USA) were diluted 1:500 into PBS, vortexed vigorously, and 200 μ l of that mixture was injected as a bolus directly in-line into the anterior segment organ culture and perfused for 1 hour. This time was adjusted to accommodate different flow rates such that a minimum threshold (100 μ l) of the tracer was perfused. Anterior chambers were then flushed thoroughly with PBS before continuing to perfuse anterior segments with medium. Flow rates were recorded over 7 days of continuous perfusion at a single pressure. A second bolus of fluorescently-labeled amine-modified 200nm Fluospheres (green) was prepared as described above, injected in-line into the anterior segment organ culture, and perfused for 1 hour (flow-adjusted time). Time points of injected fluorescent label are indicated in Fig. 1. Perfusion was stopped and anterior segments were imaged *en face* using a Leica DM500 microscope (Leica Microsystems, Buffalo Grove, IL, USA). A representative image is shown in our previous work indicating HF and LF regions (Vranka et al., 2015). The sample size of perfused eyes at normal physiologic pressure (1x or 8.8 mmHg) was N = 10 biological replicates; and the sample size of perfused eyes at elevated pressure (2x or 17.6 mmHg) was N = 6 biological replicates.

2.3. Quantitation of Flow Regions

Relative fluorescence intensity was mapped across flow regions of the TM and plotted versus circumferential distance using Image J software as documented previously (Vranka et al., 2015). In brief, individual images of the eye were merged in Photoshop using the photomerge feature creating a composite collage image of the total circumferential TM in a single color (red or green fluorescence). (Segments that did not automatically merge were done manually.) ImageJ software was then used to generate graphical output of the relative fluorescence intensity (RFU) versus circumferential distance. This was done separately for each color label (red represents the labeling at day 1, and green represents the labeling at day 8). This output consisted of approximately 10,000 data points around the circumference of the TM for each eye for each color label (red and green), and was subsequently graphed as RFU vs. distance (inches). A representative eye is shown in Fig. 2 where panel A shows the red fluorescent label on top and the graph of RFU versus distance on the bottom, panel B shows the green fluorescent label (of the same eye) with the graphical output on the bottom, and panel C shows the merged image of the two labels of the same eye. Total RFU was used to normalize the intensity of the two colors on a per eye basis and these normalized intensities were overlaid on the same graph (Fig. 2C) of RFU versus distance. High flow (HF) regions were defined as the top 1/3 of normalized RFU intensity, low flow (LF) regions were the bottom 1/3 RFU intensity, and intermediate flow (If or MF) regions were everything in between. Percent HF, MF, and LF regions were determined for the day 1 label (red) and the day 8 label (green) and the percent size change was determined for all regions. In order to determine the correlation between the red and green tracer data from individual eyes, a Pearson's correlation coefficient (r) was measured using GraphPad Prism software, and representative graphs are shown. These calculations included a $\pm 95\%$ confidence interval for each correlation coefficient and a two-tailed P value for each. In addition, cross-correlation between each pair of redgreen tracer data sets was performed using the 'xcorr'

function in MATLAB, and correlation and lag vectors are plotted against each other. Subsequently, for 6 whole globes whose perfusion experiments were paired, for example where one eye was used for 1x, and another for 2x, the cross-correlation measures were averaged and its mean $\pm$ 95% confidence interval were plotted. Further, the area under the curve for the red and green RFU values were obtained along their respective distance using the 'trapz' function in MATLAB. Subsequently, for each paired whole globe, the difference between the areas of the green to red tracers were plotted as histograms.

2.4. Statistical Analysis

Data were analyzed using Graph-Pad Prism 6 software for statistical significance using one-way ANOVA with multiple comparisons (Tukey test for multiple comparisons). The studies presented here were from an N = 10 biological replicates for the 1x pressure comparisons and N = 6 biological replicates for the 2x pressure comparisons, where a p-value of 0.05 or less is considered to be statistically significant. ANOVA with Tukey test for multiple comparisons was used to determine statistical significance of the differences in amount of each of the segmental flow regions (HF, MF or LF) in a single donor at day 1 compared with those at day 8. Differences in the segmental flow regions between contralateral eyes from the same donor were measured for statistical significance using a paired t-test (mean of the difference in the right versus the left eye, N = 8). This was done for the pairs of eyes that were both flowed at a continuous 1x perfusion pressure, as well as for those pairs of eyes in which one was flowed continuously at 1x pressure and the other eye from the same donor was flowed continuously at 2x pressure.

3. Results

3.1. Segmental flow regions measured as percent of total when perfused at physiologic (1x) pressure

Anterior segments were perfused in organ culture at physiologic pressure (1x) for 24 hours prior to labeling with a red fluorescent tracer. Perfusion was continued for 7 days prior to labeling with the green fluorescent tracer. Flow rates were measured for the duration of the perfusion period (Fig. 1). Anterior segments were imaged and segmental flow regions were quantitated for each eye (as is illustrated in Fig. 2). The graph and table in Fig. 3 show the mean values of HF, MF, and LF regions measured at day 1 and at day 8 of continuous perfusion at 1x pressure. The percentage change for each region was determined over the 7-day time period. At both the 1-day and 8-day time point the LF regions were found to predominate (47.1% and 48.4% of the total TM respectively) with relatively little change over time (Fig. 3). The MF regions were 34.2% of total TM at day-1 and 39.5% of total TM at day-8. The largest and most statistically significant change over the 7-day period of perfusion at physiologic pressure was a decrease in the HF regions by 6.3% from day 1 to day 8 (Fig. 3). This difference between the respective high flow regions at day 1 and day 8 were determined to be statistically significant (n = 10 biological replicates, using ANOVA with multiple comparisons where $p < 0.05$).

3.2. Segmental flow regions measured as percent of total when perfused at elevated (2x) pressure

Next, anterior segments were perfused in organ culture for 24 hours at 1x pressure prior to labeling with a red fluorescent tracer. Perfusion pressure was then elevated (to 2x) and perfusion was continued for 7 days at a continuously-elevated perfusion pressure (2x). A second green fluorescent tracer was perfused at the end of the pressure challenge time period (day 8). Flow rates were measured for the duration of the experiment (Fig. 1). Anterior segments were imaged and segmental flow regions were quantitated for each eye. The graph and table in Fig.4 show the mean values of HF, MF, and LF regions at day 1 and at day 8 of continuous perfusion at 2x pressure, and the percentage changes for each region over time. Again, the amount of LF regions predominated both at day 1 (57.4% of total) but decreased to 45% of total TM by day 8. The amount of HF regions was 21.9% of total TM at day 1, but decreased to only 11.7% of TM after 7 days of perfusion at elevated pressure. The largest change in amount of flow regions over 7 days of perfusion at 2x pressure was seen with the MF regions. At day 1 they were 20.7% of total TM, whereas they increased to 43.3% of total TM after 7 days of perfusion at elevated pressure. This was an increase of 22.6% and was determined to be statistically significant ($n = 6$ biological replicates, using ANOVA with multiple comparisons test where $p < 0.05$.) In addition to the increase in MF regions after 7 days of perfusion at 2x pressure, other differences were also statistically significant including the comparison between the HF and LF regions (red), as well as the differences between the MF and LF regions (red) (Fig. 4).

3.3. Variability in positional changes in distribution of segmental flow regions

No large, easily observable differences were apparent between flow regions of anterior segments from eyes that were perfused at 1x pressure (Fig. 5). The red, green, and merged images of a representative eye perfused at 1x pressure is shown (Fig. 5A–5C), along with the graphical output of each tracer in RFU versus distance (Fig. 5D) and the correlation between the red and green tracer (Fig. 5E). The Pearson's correlation coefficient of the red and green tracer signal was determined to be $r = 0.8639$ indicating a strong, positive correlation. However, in some pairs of donor eyes where one eye was perfused continuously at 1x pressure over the 7-day time period and the other eye was perfused at 2x pressure over the 7-day time period, gross differences in the MF flow regions were apparent (as is illustrated in Fig. 6). The red, green, and merged images of a representative eye perfused at 2x pressure is shown (Fig. 6A–6C), along with the graphical output of each tracer in RFU versus distance (Fig. 6D) and the correlation between the red and green tracer (Fig. 6E). The Pearson's correlation coefficient of the red and green tracer signal was determined to be $r = 0.3879$ indicating a weak, positive correlation. In some eyes these MF areas appeared as “new flow regions” where they had previously been determined to be LF regions (though not in all LF regions around the circumference of the eye) (indicated by white arrows in Fig. 6A – 6D). In some cases, the increase in MF regions were more subtle and only visible at the edges of the regions where they had previously been determined as either LF or HF regions, though this varied considerably between biological donors. In a majority of the eyes perfused at 2x pressure, the “new MF areas” were found in what had previously been LF regions. To statistically compare the shifts in RFU measures, we utilized the cross-correlation approach. Representative cross-correlation for whole globe # 0423 comparing flow in the 1x eye

(shown in Fig. 5) and the 2x eye (shown in Fig. 6) is demonstrated in Fig. 7A. A narrower graph with peak centered on '0' indicates good correlation between the RFU values for red and green tracers, as is the case for the 1x eye. A wider graph or one with multiple peaks indicates the red and green RFU values across the distance do not overlap with each other, as is the case for the 2x eye. The mean $\pm$ 95% confidence interval plots in Fig. 7B demonstrate that under 1x pressure, the differences in RFU values for red and green tracers are significantly lesser than those observed under 2x pressure, suggesting shifts in uptake of fluorescent tracers. In order to establish that there was a difference in the number of flow regions, we determined the difference in area under the curve for the red and green RFU values vs distance. Indeed, under 2x pressure there was significant increase in the difference between areas under the curve for the two tracers (Fig. 7C) for all the paired eyes, suggesting an increase in number of flow areas within the TM. This was not observed under 1x pressure consistent with our other analyses, that changes in flow areas are minimal.

3.4. Differences between contralateral eyes compared with eyes from different donors

When comparing segmental flow patterns of contralateral eyes from the same donor, the overall patterns of the segmental regions were strikingly similar in distribution and amount of flow regions per eye, although anatomical locations were not determined in individual eyes in these studies. This was true when the right and left eyes from the same donor were both perfused continuously at 1x pressure. The same was not true when comparing segmental patterns in eyes from different biological donors. To assess whether any differences between segmental flow regions within a single donor (comparing the right to the left eye) were significant, we measured the mean of the differences in segmental flow regions of contralateral eyes and using a paired t-test we found no statistically significant differences (see Supplementary Table S2). Interestingly, the mean of the differences in segmental distribution between the left and right eye from the same donor were also not statistically significant when one eye was perfused at 1x pressure and the other at 2x pressure; however, these contralateral eye comparisons are limited by a lack of anatomical location information.

4. Discussion

Although segmental or non-uniform patterns of outflow have been recognized for many years, their roles in both normal maintenance of outflow resistance, as well as in the development of glaucoma, remain elusive. To better understand the role of segmental flow we recently measured differences in the elastic moduli of segmental regions of the TM from human anterior segments perfused at normal or elevated pressure in organ culture (Vranka et al., 2018). In addition, we demonstrated that low flow regions of glaucomatous TM are far stiffer than those in normal TM (Raghunathan et al., 2018). Concomitantly, we showed that there is an increase in the amount of low flow regions and a decrease in the amount of high flow regions in glaucoma. Together these data suggest that segmental flow regions are directly affected in glaucoma.

To further develop our hypotheses that segmental flow is important for the maintenance of IOP and that segmental flow is directly affected in glaucoma, we needed insight into the

dynamic nature of the segmental flow regions. In this study we addressed the dual questions of whether these segmental flow regions are long-lived, and whether they are affected by changes in intraocular pressure. This was done by perfusing fluorescent tracers into anterior segment organ culture and measuring the amounts, and distribution, of the segmental flow regions of the TM after 7 days. This 7-day time point between the first and second labeling period is ideal, as this is more than sufficient time to obtain a strong and sustained homeostatic response. Within this timeframe, we expected turnover of the ECM to be slow (on the order of days), and therefore, the flow regions to be relatively stable. Our data indeed show that flow regions are long-lived and reasonably stable at 1x pressure over the time period tested. When we analyzed the correlation between the red and green tracer signal in individual eyes perfused at 1x pressure, we found a relatively strong positive correlation for the majority of eyes, although there was not a complete overlap between the two tracers (Pearson's correlation coefficients were always <0.9), likely due to the 1 week perfusion time between the red and green labeling or other factors. Further, we expected to see some shift in the distribution of flow regions when challenged with continuously-elevated pressure, since normal eyes are able to adjust homeostatically within that timeframe. Our data demonstrate that overall the number of areas across which active outflow occurs increases. When we analyzed the correlation between the red and green tracer signal in eyes perfused at elevated (2x) pressure, we found a weak positive correlation (Pearson's correlation coefficients <0.5) indicating a lower degree of correlation between the red and green tracer positions. This data was supported by cross-correlation analyses showing statistically significant shifts in the red and green tracer datasets as a result of elevated perfusion pressure. Also, our data suggests that there are dynamic changes during IOP homeostatic resistance adjustment in response to elevated pressure whereby the MF regions are significantly increased, while HF and LF regions are decreased. The unexpected finding regarding the dynamic nature of the MF regions in particular, in response to elevated pressure, suggests an important role in the maintenance of aqueous humor outflow over relatively short periods of time.

We did not track flow regions in relationship to anatomical markers, however, there is clearly a relationship between flow regions and collector channels, as shown previously (Cha et al., 2016; Hann and Fautsch, 2009). It follows that in glaucoma, where we've previously shown there to be larger areas of low- or no-flow regions in the TM, there may be an associated decrease in active collector channels. This would be in agreement with previous published work showing a decreased number of active collector channels in POAG eyes compared with normal eyes when perfused at elevated pressure (Hann et al., 2014). Our data presented here strongly suggest that in normal eyes there is a reliable homeostatic mechanism in place to relieve periods of elevated pressure by increasing MF regions. However, in glaucoma, where the TM is unable to homeostase in response to elevated pressure, we suspect that the LF regions are unable to revert to MF regions to alleviate the pressure conditions, ultimately causing optic nerve damage. This clearly warrants further investigation to understand the mechanisms of this adaptive feature of segmental outflow and the consequences of its malfunction in disease.

The limitations of this study are two-fold: one is the biological variability that we see from donor to donor in terms of overall amounts and distributions of the segmental flow regions

and their responses to elevated pressure. The second limitation of this study is that the anterior segment organ culture can only be used for about 1 month post-mortem. After that time the cell viability is thought to decrease to the point that the tissues can no longer regulate the outflow resistance. This is particularly true for aged donor eyes. The average age of the donor eyes used in this study is 77 years old. It will be important in future studies to measure the segmental flow regions in younger eyes. Over the lifetime of an individual it is likely that segmental regions change in response to various external stimuli, and aging may well have a profound effect. Beyond the time frames that we have used in this study the question remains: how permanent are these flow regions and under what conditions do they change?

In conclusion, changes in segmentation are of considerable interest when considering approaches to develop therapeutic targets to specific regions of the TM, for example to increase outflow in low flowing regions of the TM in glaucomatous patients. In spite of the limitations, our data suggest specific and distinct roles for HF, MF, and LF regions that can guide our understanding of normal outflow resistance. In order to determine whether various treatments may be used to alter the segmental flow regions in the TM, it was first important to determine whether these regions changed over time, both in an individual eye, as well as between contralateral eyes from a single donor. Our data here show no significant difference in the segmental distribution of contralateral eyes perfused over time from the same donor. However, it is important to point out that we did not determine the anatomical location of the segmental flow regions in individual eyes in these studies. Therefore, while we see similar amounts of segmental flow regions between contralateral eyes, we cannot conclude that these are located in anatomically similar locations. Future detailed studies with such anatomical location information will help to determine whether contralateral eyes may be used as experimental controls when determining the usefulness of potential treatments to affect segmental outflow. The insights gained from this work will help us to determine how IOP regulation and outflow segmentation is altered in glaucoma with the aim to develop targeted treatments for the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Segmental flow regions of the TM are relatively stable after 7 days of perfusion.
- Intermediate flow regions are increased after perfusion at elevated pressure.
- These dynamic changes are part of the normal homeostatic response to pressure.
- Contralateral eyes show small differences in amounts of flow regions.

FIGURE 1.

Normalized flow rates of anterior segments perfused at physiologic (1x) and elevated (2x) perfusion pressure. Flow rates were measured for the duration of the experiment. Labeling time for the first (red) and second (green) fluorescent tracer are indicated on the graph. Eyes flowed at 1x pressure showed a steady flow rate whereas, eyes flowed at 2x pressure showed a gradual increase in flow rate.

FIGURE 2.

En face images of red labeled- (A), green labeled- (B), and red and green merged (C) perfused anterior segment of a representative eye perfused at physiologic (1x) pressure. The lower portion of each panel shows the graphical output of the relative fluorescence (RFU) on the vertical axis versus distance (inches) on the horizontal axis. The RFU values were measured using image J starting from the upper left corner of the image and running clockwise around the circumference of the eye.

1x	DAY 1 (Red)	DAY 8 (Green)	% Change
HF	18.6 ± 2.2	12.3 ± 2.7	-6.3
MF	34.2 ± 6.5	39.5 ± 7.5	+5.3
LF	47.1 ± 6.9	48.4 ± 8.4	-1.3

FIGURE 3.

Segmental flow regions of the TM from anterior segments continuously perfused at physiologic (1x) pressure over 7 days. Red fluospheres (“red”) were perfused at day 1 and green fluospheres (“grn”) were perfused at day 8. Relative fluorescence was measured for each color and amount of segmental flow regions were compared. Bars represent mean values $\pm$ s.e.m. of high (HF), intermediate (MF) and low (LF) flow regions as a percentage of the total TM for each color; ANOVA with multiple comparisons where * $p < 0.05$, (N = 10). Mean values, as shown in table below the graph, were determined as percent of total circumferential flow $\pm$ standard error of the mean (sem). The change in percent of flow regions was -6.3 (n.s.) for HF regions, $+5.3$ (n.s.) for MF regions and -1.3 (n.s.) for LF regions when compared with those same regions at day 1, respectively. “n.s.” = not significant; ANOVA with multiple comparisons where $p < 0.05$ is statistically significant (N = 10).

2x	DAY 1 (Red)	DAY 8 (Green)	% Change
HF	21.9 ± 4.3	11.7 ± 3.5	- 10.2
MF	20.7 ± 3.7	43.3 ± 5.2	+ 22.6*
LF	57.4 ± 5.5	45.0 ± 7.2	- 12.4

FIGURE 4.

Segmental flow regions of the TM from anterior segments continuously perfused at elevated (2x) pressure over 7 days. Red fluospheres (“red”) were perfused at day 1 and green fluospheres (“grn”) were perfused at day 8. Relative fluorescence was measured for each color and amount of segmental flow regions were compared. Bars represent mean values $\pm$ s.e.m. of high (HF), intermediate (MF) and low (LF) flow regions as a percentage of the total TM for each color; ANOVA with multiple comparisons where * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$ ($N = 6$). Mean values, as shown in table below graph, were determined as percent of total circumferential flow $\pm$ standard error of the mean (sem). There are significantly more MF regions after perfusion at 2x pressure for 7 days. The change in percent of flow regions was -10.2 (n.s.) for HF regions, $+22.6$ (* $p < 0.05$) for MF regions and $+12.4$ (n.s.) for LF regions when compared with those same regions at day 1, respectively; * $p < 0.05$; ANOVA with multiple comparisons ($N = 6$).

FIGURE 5.

En face images of the same eye illustrate the distribution of segmental flow regions at day 1 (red) and day 8 (green) of perfusion at 1x pressure. (A) Image of the day 1 label (red), (B) day 8 label (green), and (C) red and green images are merged. Yellow areas represent areas where red and green labels are present at approximately equal levels. Not all eyes showed similar patterns of distribution. (D) Graphical output of the red and green relative fluorescence (RFU) on the vertical axis versus distance (in inches) on the horizontal axis overlaid in a single graph. Blue shaded area indicates LF, green shaded area indicates MF, and pink shaded area (upper portion) indicates HF regions as was determined for both labels. (E) The correlation of the red versus green signal around the circumference of the eye was calculated and graphed; Pearson's correlation coefficient (r) = 0.8639, where $r^2 = 0.7463$, ($p < 0.0001$).

FIGURE 6.

En face eye images illustrate the change in distribution of segmental flow regions at day 1 and day 8 perfusion at 2x pressure. (A) Day 1 label shows large upper portion of LF region (white arrows). (B) Day 8 label shows the same area that has become MF after 8 days of perfusion at 2x pressure (white arrows). (C) Red and green images are merged. Yellow areas represent areas where red and green labels are present at approximately equal levels. Not all eyes showed similar patterns of distribution. (D) Graphical output of the red and green relative fluorescence (RFU) on the vertical axis versus distance (in inches) on the horizontal axis overlaid in a single graph. Blue shaded area indicates LF, green shaded area indicates MF, and pink shaded area (upper portion) indicates HF regions as was determined for both labels. Corresponding areas of "new MF areas" are indicated by white arrows on the images and graph (A-D). (E) The correlation of the red versus green signal around the circumference of the eye was calculated and graphed and shows a relatively nonlinear association; Pearson's correlation coefficient (r) = 0.0.3879, where $r^2 = 0.1505$, ($p < 0.0001$).

FIGURE 7.

(A) Cross-correlation data between the red and green tracers for whole globe# 0423 where the 1x eye (blue line) and the 2x eye (red line), as illustrated in Fig. 5 and 6, respectively, demonstrate a mismatch or shift in the number of flow regions under elevated pressure as indicated by multiple peaks at varying distance (lag) from 0 (black arrows).

(B) Mean $\pm$ 95% confidence interval in cross-correlation measures comparing 6 pairs of eyes (1x eyes on the left panel and 2x eyes on the right) demonstrate that under elevated pressure, the plot is wider, shorter, and with the appearance of additional peaks (black arrows). The large values of 95% confidence interval demonstrate donor-to-donor variability in homeostatic response. (C) A greater difference in area under the curves comparing red and green tracers correspond to the increase in number of effective flow regions under elevated pressure, which was clearly absent under 1x pressure.