

# 1 Quantification of Focal Outflow Enhancement using 2 Differential Canalograms

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4 Ralitsa T. Loewen,<sup>1</sup> Eric N. Brown,<sup>2</sup> Gordon Scott,<sup>1</sup> Hardik Parikh,<sup>1,3</sup> Joel S. Schuman,<sup>1,4</sup> Nils A.  
5 Loewen<sup>1\*</sup>

6  
7 1 Department of Ophthalmology, University of Pittsburgh Medical Center, Pittsburgh, PA

8 2 Department of Ophthalmology, Vanderbilt University School of Medicine, Nashville, TN

9 3 School of Medicine, Rutgers State University of New Jersey, New Brunswick, NJ

10 4 School of Medicine, New York University, New York, NY

11 \* Corresponding author

12 Nils A. Loewen, MD, PhD

13 Department of Ophthalmology

14 University of Pittsburgh

15 203 Lothrop, Suite 819

16 Pittsburgh, PA 15213

17 loewen.nils@gmail.com

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## 38 Abstract

39 **Purpose:** To quantify regional changes of conventional outflow caused by ab interno

40 trabeculectomy (AIT).

41 **Methods:** Gonioscopic, plasma-mediated ab interno trabeculectomy (AIT; Trabectome, Neomedix,

42 Tustin, CA) was established in enucleated pig eyes. We developed a program to automatically

43 quantify outflow changes (R, package eye-canalogram, github.com) using a fluorescent tracer

44 reperfusion technique. Trabecular meshwork (TM) ablation was demonstrated with fluorescent

45 spheres in 6 eyes before formal outflow quantification with two dye reperfusion canalograms in 6

46 further eyes. Eyes were perfused with a central, intracameral needle at 15 mmHg. Canalograms

47 and histology were correlated for each eye.

48 **Results:** The pig eye provided a model with high similarity to AIT in human patients. Histology

49 indicated ablation of TM and unroofing of most Schlemm's canal segments. Spheres highlighted

50 additional circumferential and radial outflow beyond the immediate area of ablation. Differential

51 canalograms showed that AIT caused an increase of outflow of  $17\pm 5$  fold inferonasally (IN),  $14\pm 3$

52 fold superonasally (SN) and also an increase in the opposite quadrants with a  $2\pm 1$  fold increase

53 superotemporally (ST) and  $3\pm 3$  inferotemporally (IT). Perilimbal specific flow image analysis

54 showed an accelerated nasal filling with an additional perilimbal flow direction into adjacent

55 quadrants.

56 **Conclusion:** A quantitative, differential canalography technique was developed that allows to

57 quantify supraphysiological outflow enhancement by AIT.

## 58 Introduction

59 Bulk outflow of aqueous humor and its relationship to intraocular pressure (IOP) has been  
60 well characterized.<sup>1,2</sup> In contrast, focal outflow and enhancement of focal outflow by  
61 microincisional glaucoma surgeries has only been modeled mathematically<sup>3,4</sup> but not been  
62 measured directly. Tracers have been used to highlight areas of increased flow through the TM  
63 where they become lodged near collector channel openings.<sup>5,6</sup> The TM and the aqueous spaces of  
64 Schlemm's canal and collector channels in the superficial to mid-level sclera can also be imaged  
65 non-invasively by spectral domain optical coherence tomography (SD-OCT).<sup>7</sup> Wang et al used a  
66 Doppler strategy to detect movements of gold nanorods by SD-OCT but a quantification of flow  
67 could not be obtained.<sup>8</sup> This approach may have limited use in vivo due to toxicity.<sup>9</sup>

68 It is estimated that the smallest of the current trabecular meshwork micro-bypass implants  
69 is limited to drainage segments of about 60 degrees,<sup>10,11</sup> while larger ones access additional clock  
70 hours.<sup>12</sup> Trabecular meshwork ablation can provide more extensive angle access by allowing to  
71 skip areas of discontinuity of Schlemm's canal (SC).<sup>11,13</sup> Although the trabecular meshwork is  
72 thought to be the anatomic location of primary outflow resistance,<sup>14</sup> intraocular pressures that  
73 are close to the theoretical limit of episcleral venous pressure<sup>11</sup> are rarely achieved with trabecular  
74 bypasses<sup>15</sup> or with trabecular ablation.<sup>16</sup>

75 Here, we hypothesized that it is possible to develop a differential canalography technique  
76 to directly analyze areas of altered outflow before and after an intervention. We refined recently  
77 introduced methods of quantitative canalography<sup>17</sup> and used them to measure conventional  
78 outflow enhancement following plasma-mediated ab interno trabeculectomy.

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## 81 Methods

### 82 **Trabectome-Mediated Ab Interno Trabeculectomy in Pig Eyes**

83 Pig eyes were obtained from a local abattoir. Only eyes that could be identified as right eyes were  
84 used. Within 2 hours of death eyelids and adnexal structures were excised, while the conjunctiva  
85 was preserved for the entire length of the globe. Eyes irrigated with phosphate buffered saline  
86 (PBS) were placed with the optic nerve into a cryogenic vial cup (CryoElite Cryogenic Vial  
87 #W985100, Wheaton Science Products, Millville, NJ) for compression free mount as described  
88 before.<sup>17</sup> Similar to AIT in human eyes (Figure 1, A),<sup>11</sup> eyes were positioned under a surgical  
89 microscope looking up with the temporal side of the eye directed towards the surgeon. A clear  
90 corneal incision was fashioned with a 1.8 mm keratome approximately 2 mm anterior to the  
91 temporal limbus while the inside was slightly flared for a striae free visualization during the  
92 procedure. Eyes were then tilted 30 degrees away from the surgeon and a goniolens (Trabectome  
93 Goniolens ONT-L, #600010, Neomedix Inc., Tustin, CA) was placed on the cornea to visualize the  
94 chamber angle (Figure 1, B). The tip of a Trabectome handpiece (handpiece #600018, Neomedix  
95 Inc., Tustin, CA) that was connected to a standard Trabectome system (Trabectome System  
96 #600026, Neomedix Inc., Tustin, CA) was inserted and advanced to the opposite chamber angle.  
97 After gentle goniosynechiolysis with the side of the instrument's tip to disengage pectinate  
98 ligaments, TM ablation was continued towards the left by 45 degrees. The tip was then turned  
99 around inside of the eye, the TM was engaged again, and ablation continued by 45 degrees in the  
100 opposite direction. The instrument was withdrawn and the incision sealed with a drop of  
101 cyanoacrylate.

## 102 **Histology**

103 Segments were removed from the perfusion dish, rinsed in PBS, cut into quarters and fixed with  
104 4% paraformaldehyde and PBS for 48 hours before being placed in 70% ethanol. A corneoscleral  
105 wedge was taken from the lateral side near the incision and one from the nasal side where the ab  
106 interno trabeculectomy had been performed (Figure 2). The section was paraffin-embedded for  
107 histological processing, cut at 6  $\mu\text{m}$  thickness, and stained with hematoxylin and eosin (H&E).

## 108 **Microsphere Canalograms**

109 Fluorescent microsphere canalograms were obtained in six eyes. Microspheres (FluoSpheres  
110 Carboxylate-Modified Microspheres, 0.5  $\mu\text{m}$ , yellow-green fluorescent (505/515), 2% solids,  
111 Thermo Fisher Scientific, Eugene, OR) were tested for a size that would not pass through the TM  
112 (Figure 3, A) while allowing visualization of the collector channels (Figure 3, B). The fluorescent  
113 microspheres were diluted 100-fold with phenol red free Dulbecco's modified Eagle's media  
114 (DMEM) to make the perfusate. Following the intervention described below, ab interno  
115 trabeculectomy (AIT), a 30 gauge needle was inserted through the nasal cornea 2 mm anterior to  
116 the limbus with the tip of the needle positioned in the center of the anterior chamber. Flow was  
117 began and gravity-based infusion ensued. Fluorescence was visualized with a stereo dissecting  
118 microscope equipped for fluorescent imaging (Olympus SZX16 with GFP filter cube and DP80  
119 Monochrome/Color Camera; Olympus Corp., Center Valley, PA). Images were acquired every 20  
120 seconds for 15 minutes for time lapse analysis (CellSens, Olympus Life Science, Tokyo, Japan) with  
121 a resolution of the 2x2 binned image capture of 580 x 610.

122

## 123 **Differential Canalograms**

124 A fluorescent tracer reperfusion technique was used in six eyes to compare outflow changes  
125 before and after AIT detailed below using a quantitative canalography method we recently  
126 developed.<sup>17</sup> Whole pig eyes were prepared and mounted under a surgical microscope as  
127 previously described. A 30 gauge needle was inserted through the nasal cornea as previously  
128 described and used to remove 0.2 mL of anterior aqueous humor from the whole eye's anterior  
129 segment. A 30 gauge needle was then placed in the anterior segment using the exact same entry  
130 site and media with fluorescein (AK-FLUOR 10%, Fluorescein injection, USP, 100 mg/ml, NDC  
131 17478-253-10, Akorn, Lake Forest, IL) at a concentration of 0.017 mg/ml was infused via gravity.  
132 The outflow pattern was imaged every 20 seconds for 15 minutes using a stereo dissecting  
133 microscope equipped for fluorescent imaging (Figure 4). Fluorescein flow was then stopped and  
134 the needle was removed. Following AIT, a new 30 gauge needle was placed in the anterior  
135 segment using the same entrance wound, and media with 0.28 mg/mL Texas Red (Sulforhodamine  
136 101 acid chloride, 10 mg, Alfa Aesar, Ward Hill, MA) was subsequently infused via gravity. Again  
137 the outflow pattern was imaged every 20 seconds for 15 minutes. The eyes were then fixed and  
138 sent for histology.

139 To determine differences in chromophore detection sensitivity, a hemocytometer chamber  
140 was filled with 10  $\mu$ L each of Fluorescein and Texas Red at the previously stated concentrations.  
141 Proper exposure times of 15 ms for Fluorescein and 10 ms for Texas Red were determined with  
142 the fluorescence equipped stereo dissecting microscope above.

143 Initially, six eyes were perfused with fluorescein first immediately followed by Texas Red  
144 without AIT. Another six eyes were perfused with Texas Red first followed by fluorescein to  
145 determine whether the order of perfusion would have any effect. When there was no statistically  
146 significant difference in filling times between the two ( $p=0.06$ ,  $n=12$ ), but a 19% slower filling rate  
147 for Texas Red, the order was chosen to be fluorescein followed by Texas Red for all eyes in order  
148 to avoid false positive flow enhancement by AIT.

#### 149 **Quantification of Outflow Change**

150 As described before,<sup>17</sup> we used a program written in R<sup>18</sup> to automatically compute the focal  
151 outflow changes (Figure 5, left) and convergent perilimbal aqueous flow (Figure 5, right) using the  
152 eye-canalogram package to process the image datasets, the source code of which we made  
153 available for download (R package “eye-canalogram”, [https://github.com/enbrown/eye-](https://github.com/enbrown/eye-canalogram/tree/06461498c8)  
154 [canalogram/tree/06461498c8](https://github.com/enbrown/eye-canalogram/tree/06461498c8)).

155 Briefly, for each image set, the cornea was first manually segmented. The image resolution  
156 was then reduced to 32 x 32 macropixels per image. Generalized additive models (GAM) were fit  
157 for each macropixel. Metrics from the fit were recorded and graphically displayed (Supplemental  
158 File 2). In dot plot images, larger pixels corresponded to more intense fluorescein signals while red  
159 dots corresponded to faster filling. In addition, all regions (clock hour and three radial rings) were  
160 fit to a single GAM using smoothing terms for the radial ring, clock hour, and frame number. Pre-  
161 and post-treatment image sets for each eye were registered using the clock hour and radial

162 distance to compute the change in fit metrics (Figure 6A). Similarly, all eyes were warped to a  
163 common reference frame and averaged to produce Figure 6B.

164 Each quadrant was also analyzed for first detection of fluorescence in an outflow structure  
165 downstream of Schlemm's canal segments. Timestamps from time lapse recordings were  
166 summarized as graphs (average  $\pm$  standard deviation (SD)) for the inferonasal (IN), superonasal  
167 (SN), superotemporal (ST), and inferotemporal (IT) quadrants.

168

## 169 Results

170 Visualization and TM ablation could be achieved with a standard trabectome system and the kit-  
171 included, modified Swan Jacob gonioscopes (Figure 1, A). Pectinate ligaments had to be gently lysed  
172 (Figure 1, B, left) before the TM could be engaged for ablation (Figure 1, B, right). Power and  
173 aspiration settings were identical to surgery in human patients. During ablation, the footplate  
174 encountered more stops than typical for human eyes when SC segment ends were reached.

175 The histological analysis showed that the relatively prominent TM and common SC  
176 segments had been ablated with a few exceptions of SC segments too small to enter with the  
177 trabectome tip (Figure 2, eye 1, shown to scale). No coagulative damage was observed.

178 Eyes without AIT that were infused with fluorescent spheres showed fluorescence only in  
179 the TM. When those were hemisected and vitreous and iris removed, sphere distribution  
180 appeared relatively even throughout the TM circumference (Figure 3 A). In contrast, canalograms  
181 with fluorescent spheres obtained after AIT experienced fast filling of proximal and distal parts of  
182 the outflow system along the ablation site followed by circumferential and then centrifugal filling  
183 of adjacent quadrants. Filling of nasal quadrants was nearly ten times faster nasally than that of  
184 temporal quadrants (Figure 3 B).



185 Differential canalograms, obtained before and after AIT (Figure 4 and Supplemental File 1:  
186 Movie with parallel canalograms pre- and post-AIT) had a  $17\pm 5$  fold increase in filling inferonasally,  
187  $14\pm 3$  fold increase superonasally and also an increase in the adjacent quadrants with a  $2\pm 1$  fold  
188 increase superotemporally and  $3\pm 3$  inferotemporally. The superonasal quadrant was the fastest to  
189 fill ( $p < 0.5$ ) followed by the inferonasal and super- and inferotemporal quadrants (Figure 4 and  
190 Figure 7). Although fluorescent dyes used in these differential canalograms were not blocked by  
191 the TM like the fluorescent spheres, superotemporal and inferotemporal quadrants often filled  
192 circumferentially from the site of AIT.

193 Individual fit curve analysis indicated a shift in filling speed towards the nasal side and an  
194 overall faster filling throughout the remainder of the outflow system in most eyes (Figure 5). This  
195 can be seen by a shift towards red in the color coded analysis indicating a shorter time towards  
196 peak fluorescence, as well as the increased bubble size that describes the peak fluorescence  
197 obtained. When perilimbal flow was examined specifically, circumferential filling was evident.  
198 With the exception of eye 1, all eyes post-AIT showed an initial uptake centered on the inferonasal  
199 and superonasal quadrants as expressed by the red color coding that spreads circumferentially  
200 from here (Figure 5).

201 Changes from pre- to post-AIT fluorescence intensity changes are summarized in Figure 6.  
202 In average of all six eyes, peak intensity increased in the inferonasal quadrant and in the  
203 superonasal quadrant as well as in the remainder of the circumference (Figure 6 left). In contrast,  
204 flow rate changes from pre- to post-AIT are more substantial in the nasal quadrants.

205 These changes from pre- to post-AIT were statistically significant in all quadrants but more  
206 pronounced in the inferonasal and superonasal quadrant. Those quadrants remained significantly  
207 faster than the superotemporal and inferotemporal quadrants. There was no difference between  
208 the superotemporal and inferotemporal quadrant before or after AIT.

## 209 Discussion

210 Focal changes of conventional aqueous humor outflow are difficult to examine<sup>8,19</sup> yet are crucial to  
211 better understand how elements downstream of the TM may influence outflow. The lack of an  
212 inexpensive, readily available and high quality outflow model has impeded glaucoma research and  
213 training of surgery on this submillimeter structure alike. Here, we address both with a differential  
214 canalography technique that allows to examine the effect of trabectome-mediated AIT on focal  
215 outflow in porcine eyes.

216 Visualization of the chamber angle and ablation of TM by AIT was quite similar to surgery in  
217 human patients. Angle surgery can be practiced in human eyes<sup>20</sup> but costs of those are high and  
218 the corneal clarity is often too compromised to see the angle.<sup>21</sup> As a result, artificial eyes were  
219 preferred by trainees.<sup>21</sup> The histology following AIT shows that despite the anatomical differences  
220 between the pig and human chamber angle, an extensive ablation of TM can be achieved without  
221 an obvious coagulative thermal effect on adjacent tissues. Different from coagulation devices, the  
222 instrument used here generates plasma to ionize tissue and has a highly confined heat dissipation  
223 cone that is similar to photodisruptive lasers.<sup>11</sup>

224 The initial canalograms obtained with fluorescent microspheres demonstrated that TM  
225 must be removed before they can enter the conventional outflow system. Although the angular  
226 aqueous plexus of pigs does not have a continuous Schlemm's canal as primate eyes do, but rather  
227 multiple SC-like segments, we observed circumferential flow that extended far beyond the  
228 ablation site. This indicates that canal segments are connected and that supraphysiological flow  
229 from the site of ablated TM is displacing the normal flow that is still occurring through the non-  
230 ablated TM.

231 The perilimbal flow analysis of differential canalograms showed high flow areas near the  
232 inferonasal and superonasal angle. This matches the areas in between the recti muscles, where

233 larger collector channels reside. The circumferential flow patterns observed here contradicts the  
234 assumption of noncontinuous SC segments in the pig. A three dimensional reconstruction at a  
235 higher resolution than conventional histological sections by SD-OCT may be necessary. Such an  
236 approach has enabled discovery of previously overlooked valve-like elements in human eyes.<sup>22,23</sup>

237 We provide a heatmap summary image that combines fluorescence and flow rate of all six  
238 eyes. This allows to visualize how flow can be enhanced in non-glaucomatous eyes to levels above  
239 the physiological flow rate and beyond the TM ablation area. In this constant pressure perfusion  
240 system AIT led to increased flow and peak fluorescence in all quadrants, beyond the nasal site  
241 where AIT was performed. This has practical implications for patient care and suggests that it may  
242 not be necessary to obtain a very extensive ablation or circumferential access to the outflow tract.  
243 Carefully observing glaucoma surgeons have previously described fluid waves of saline displacing  
244 blood in collector channels after AIT.<sup>24</sup> These appeared to be limited to the site of ablation when  
245 observed through an operating microscope. Such visualization may relatively underestimates the  
246 amount of flow that could be detected with more sensitive, fluorescent dyes as used here.

247 There are well established angiography methods for organs larger than the anterior  
248 segment of the eye, such as the heart.<sup>25</sup> Characteristic for such angiography is that vessels branch  
249 off large primary vessels to subsequently smaller ones. The outflow tract of the eyes is different  
250 and has a much more diffuse and connected nexus of vessels with variable caliber. The drainage  
251 system just distal to the outer wall of the SC is more similar to the honeycomb pattern of a  
252 capillary network but its vessels are larger and collapsible depending on the perfusion pressure.  
253 This makes it challenging to use Doppler strategies to compute flow speeds in single vessels. The  
254 data presented here validates the use of fluorescence change<sup>17</sup> as a surrogate to tracking  
255 reflective particles.

256

257 In conclusion, we developed a method to quantify outflow enhancement from plasma-mediated  
258 ab interno trabeculectomy and show that nasal ablation of trabecular meshwork increased  
259 outflow not only locally but circumferentially. Methods and code provided here will aid further  
260 investigations into segmental outflow changes.

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## Legends

**Figure 1:** Trabectome-mediated ab interno trabeculectomy in porcine eyes. A) (1) Trabectome inserted through a clear corneal incision ablates TM that is engaged in between footplate and bipolar electrodes. (2) Handpiece and magnified view of tip. (3) Stand, operating console with peristaltic pump and high frequency generator and footswitch. B) Direct, gonioscopic view of ablation in porcine eye immediately before engaging the TM (left) and with tip obscured by TM during ablation (right).

**Figure 2:** Histology of ablated, nasal TM and normal, temporal TM. Eye numbers match eyes in other figures throughout manuscript. Insert: trabectome tip shown to scale. TM= trabecular meshwork, SC= Schlemm's canal, CB= ciliary body.

**Figure 3:** Microsphere canalograms before and after ab interno trabeculectomy (AIT). A) Perfusion of eye with fluorescent microspheres that cannot pass through the TM in a control eye (top left, inside view after removal of uvea and iris) but light up the nasal collector channel and beyond due to circumferential flow. B) Preferentially nasal outflow system filling is observed (anterior chamber subtracted using baseline). Circumferential filling can be seen (see also movie in Supplemental File 1). C) Short filling times after AIT nasal quadrants with occasional flow into adjacent quadrants. ST and IT filling times without non-filling eyes was  $67 \pm 22$  seconds and  $46 \pm 29$  seconds, respectively.

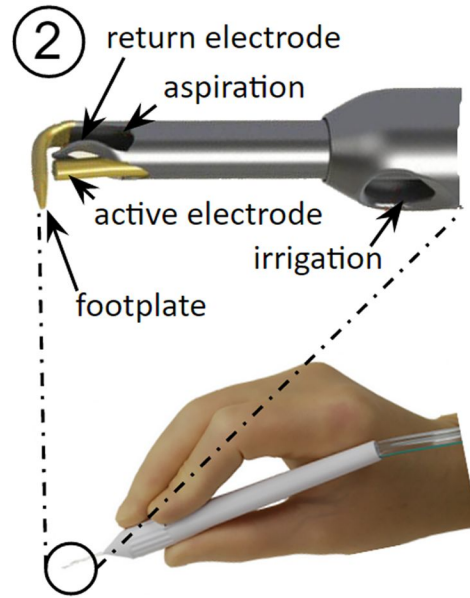
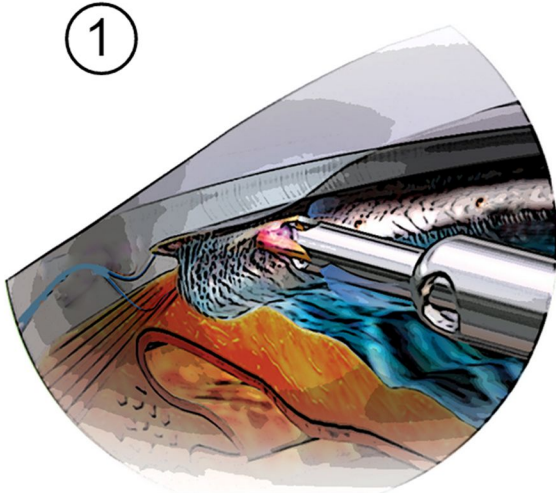
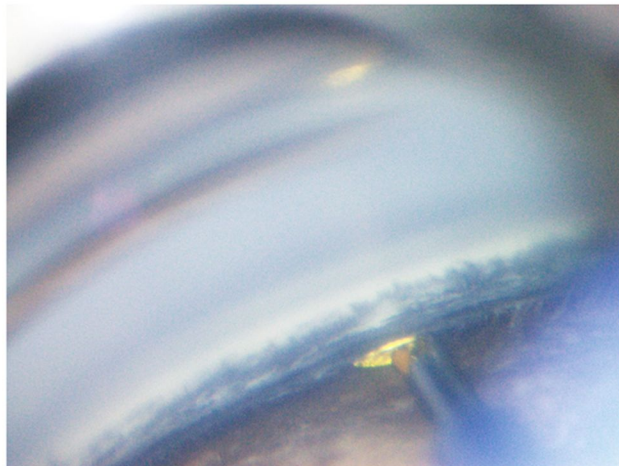
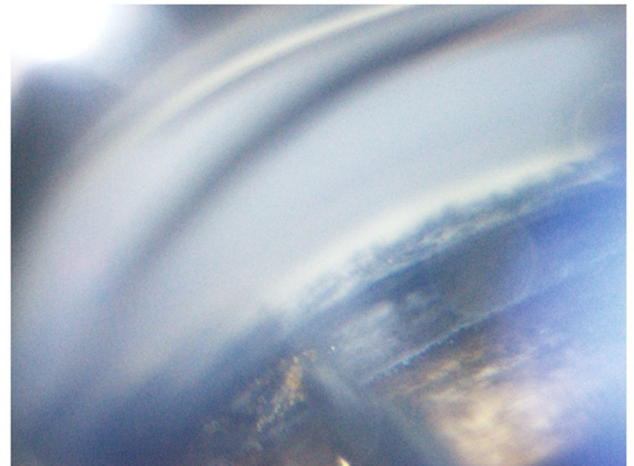
**Figure 4:** Time lapse of differential canalograms for each eye. Images are subtracted from baseline to suppress nonspecific fluorescence. Frames selected show filling of new segments. Pre- and post-ab interno trabeculectomy canalogram frames are matched (also see movie for supplemental File movie 1).

**Figure 5:** Quantitative canalograms with individual intensity fits (left) and circumferential flow rates (right). Both are increased in most eyes, even in quadrants away from the nasal AIT site.

**Figure 6:** A) Quantitative change analysis AIT. Each eye produces a pair of images: change in fluorescence intensity (left image) and change in rate of fluorescence uptake (right image). Red (positive values) indicates greater intensity or rate of uptake following AIT; blue (negative values) indicates less intensity or reduced uptake. B) Summarizing graph of intensity and rate change for all six eyes.

**Figure 7:** Outflow change summary after AIT. Short bars indicate fast filling not only in the nasal quadrants (IN: inferonasal, SN: superonasal) but also in the temporal quadrants (ST: superotemporal, IT: inferotemporal) following AIT.



**A****B****engaging****ablating**

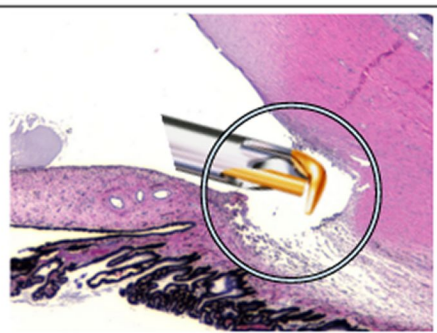
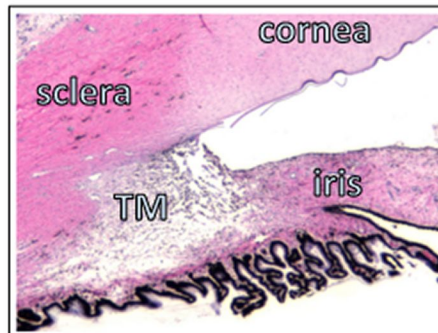
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nasal, ablated

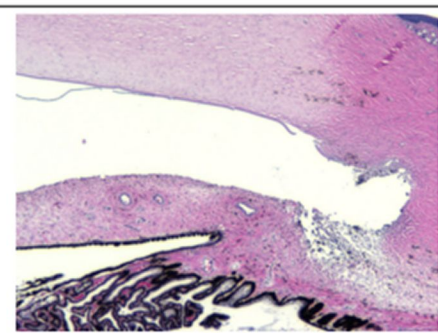
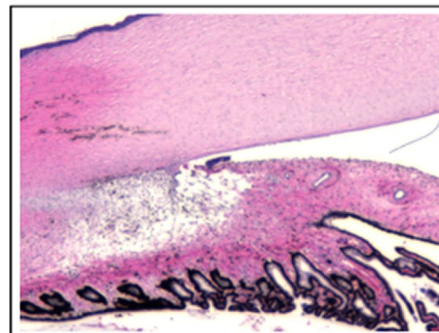
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nasal, ablated

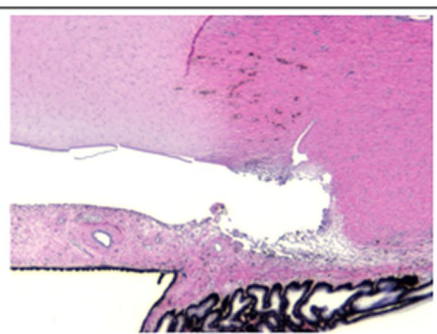
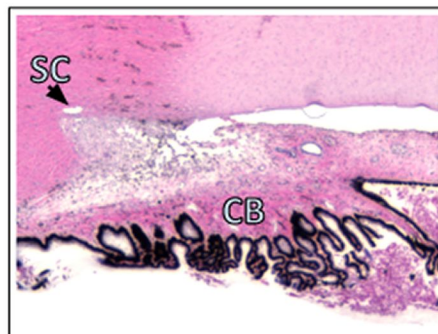
eye 1



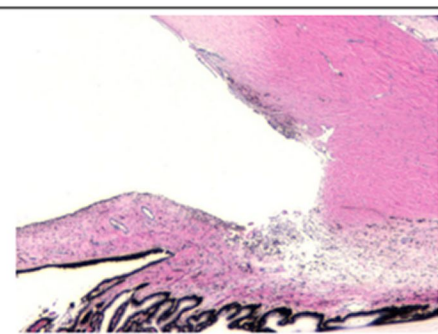
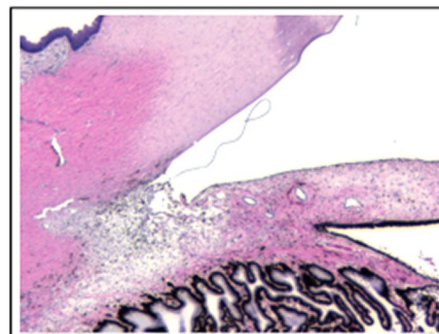
eye 4



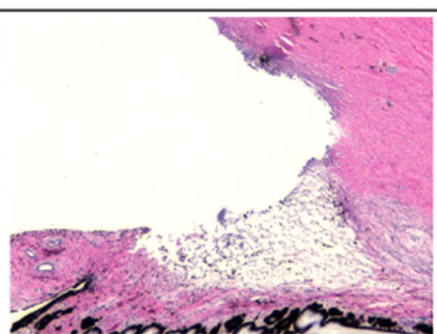
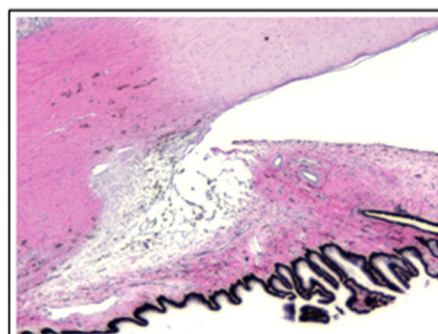
eye 2



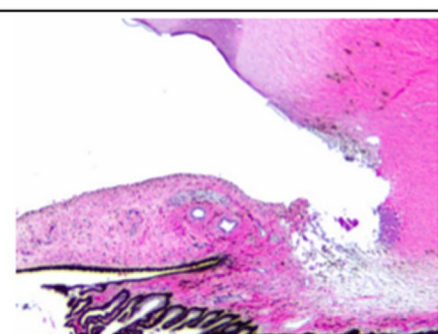
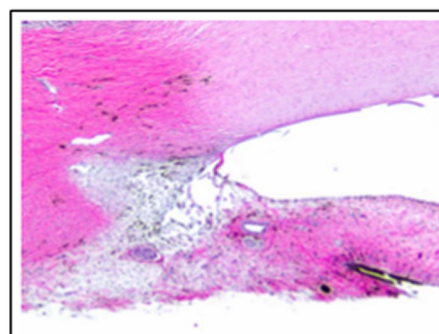
eye 5



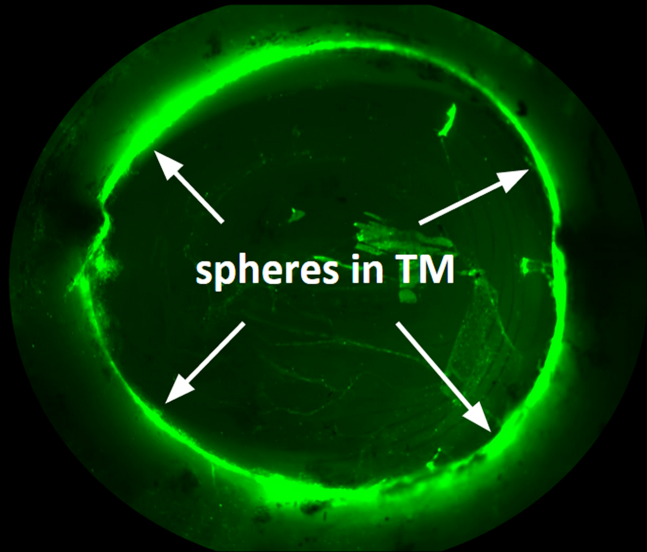
eye 3



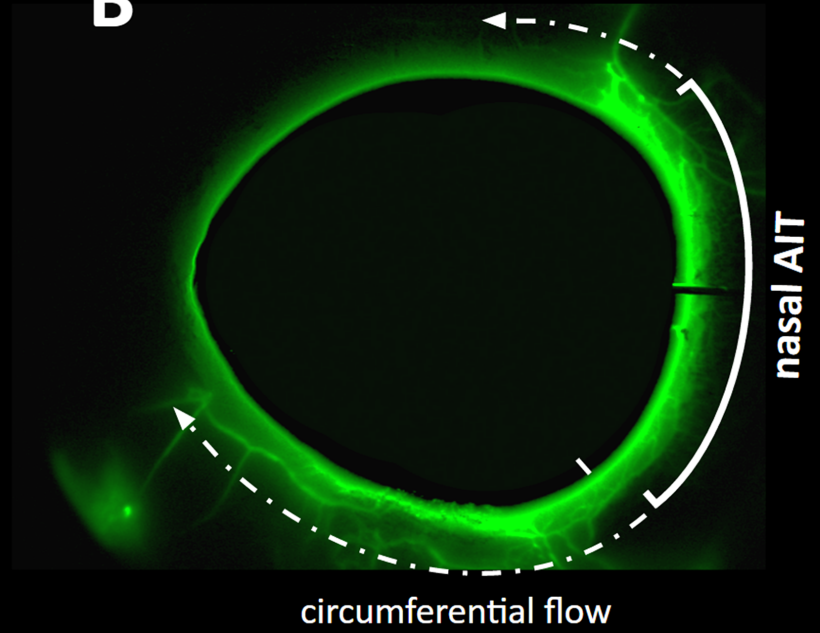
eye 6



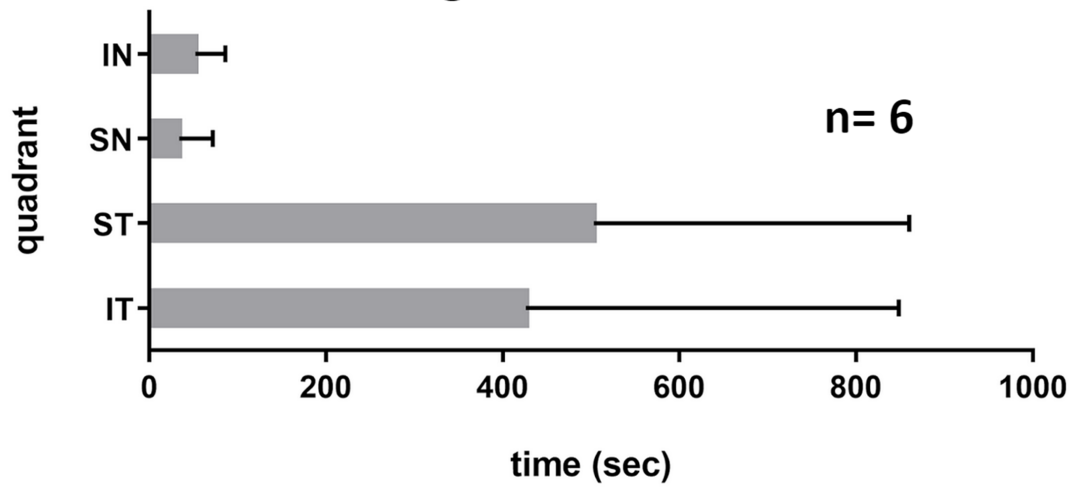
**A** anterior chamber flat mount  
no AIT

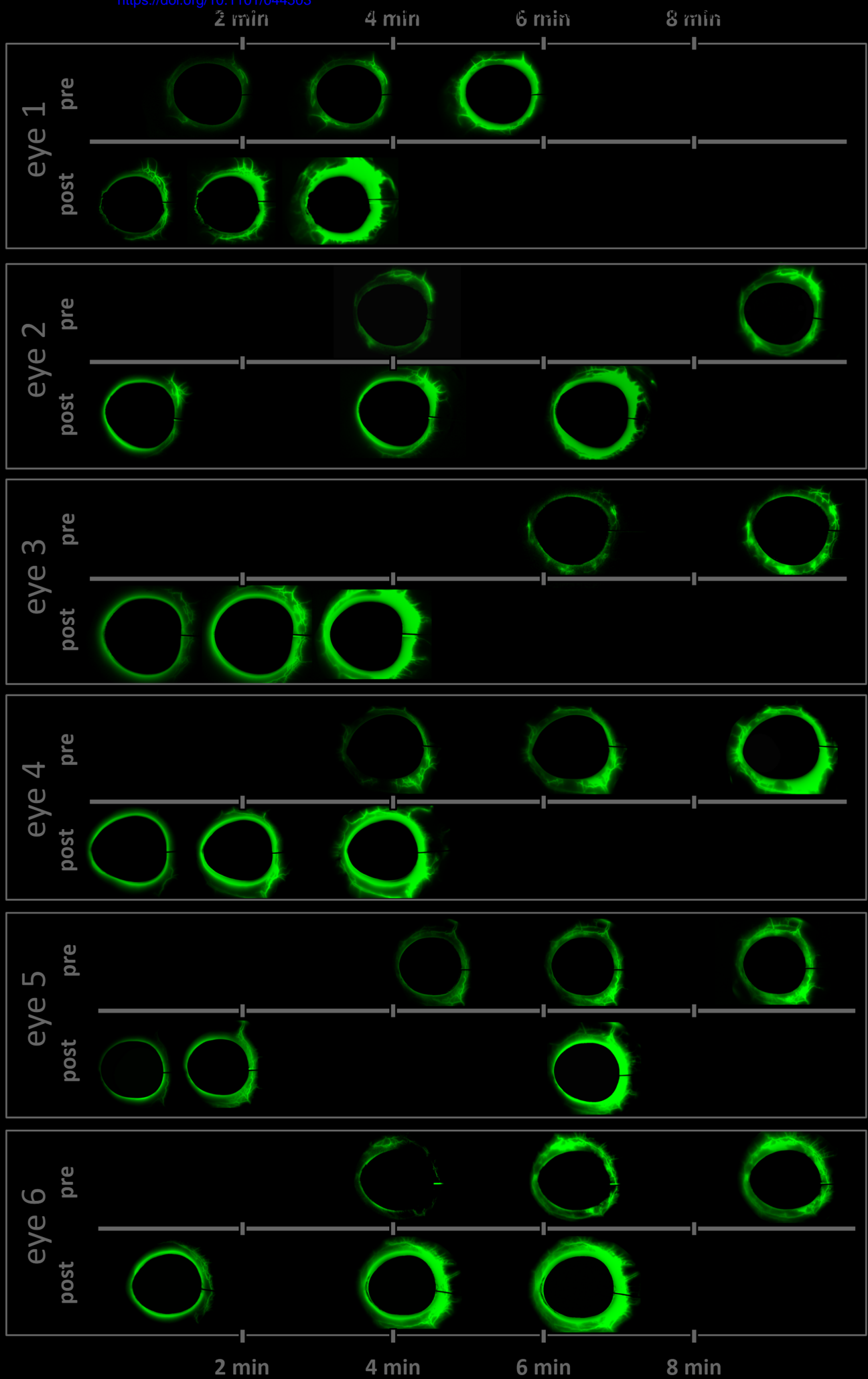


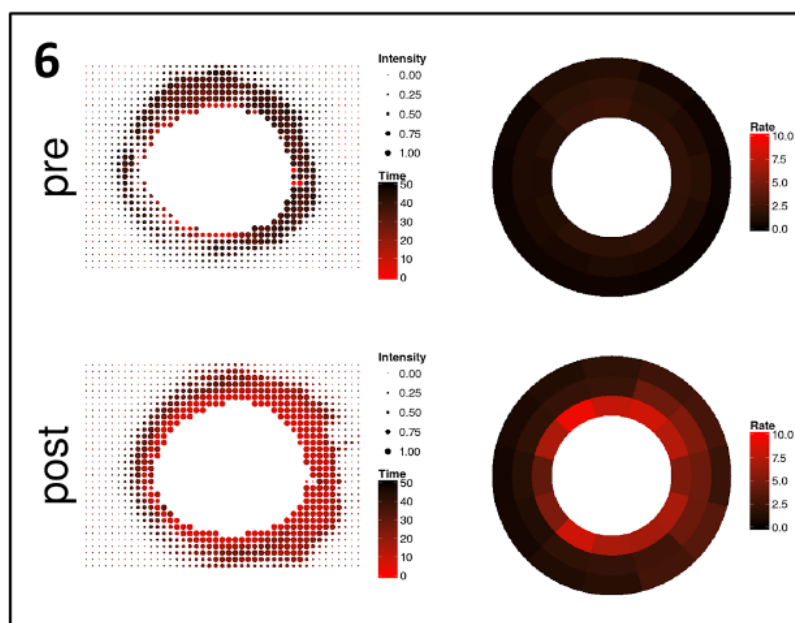
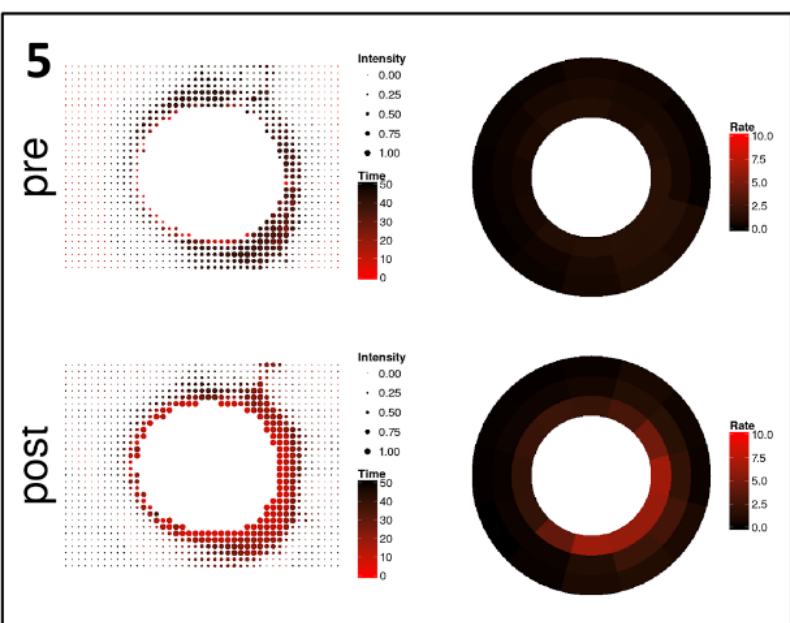
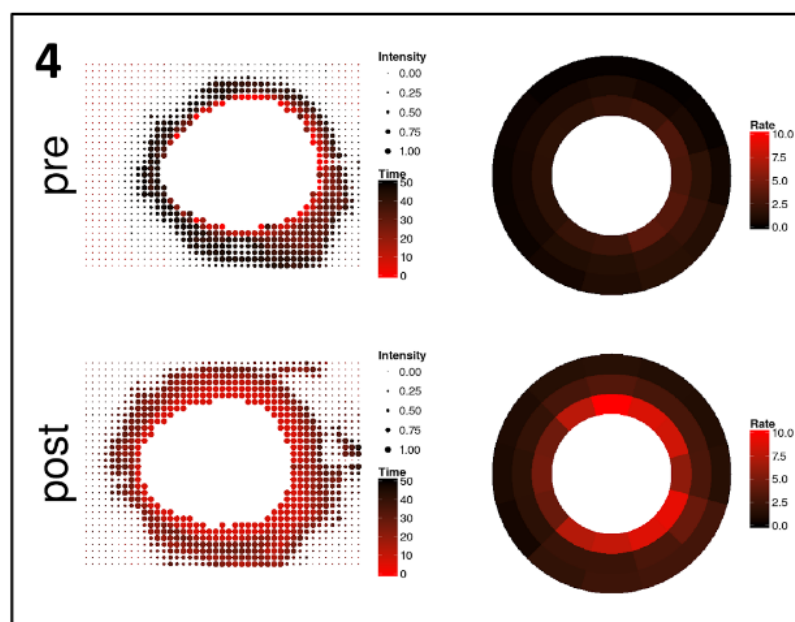
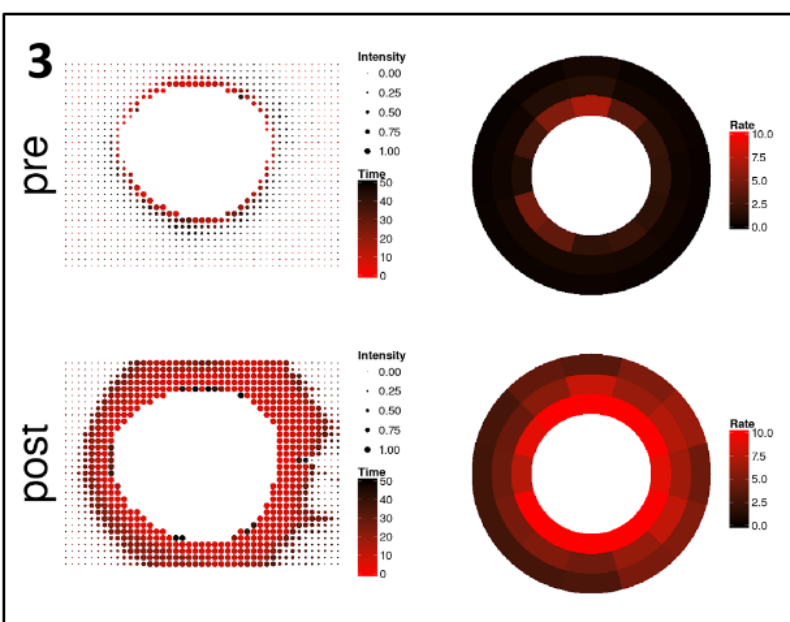
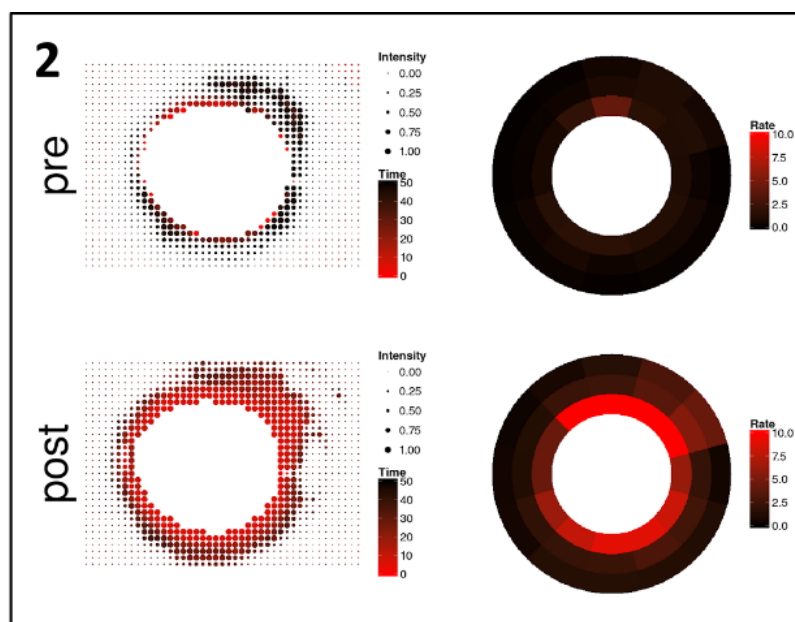
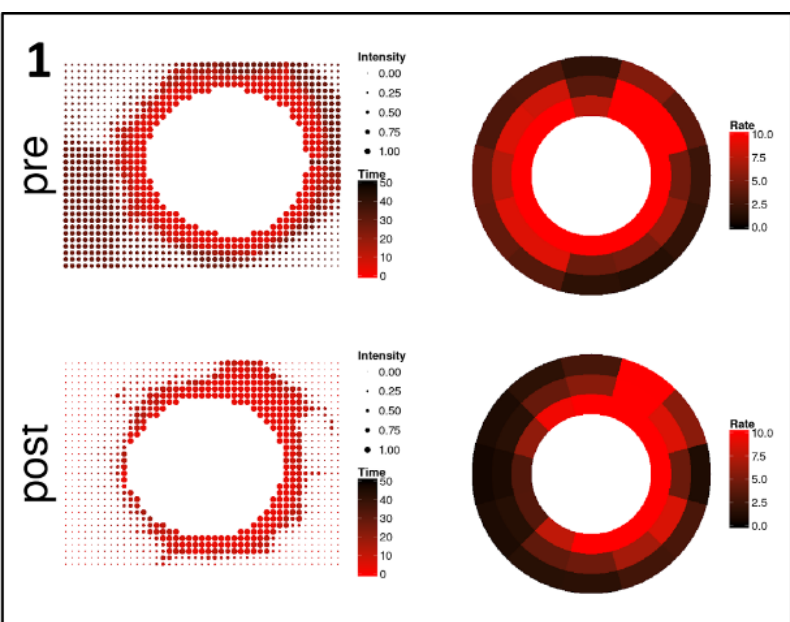
**B** sphere-canalogram after AIT



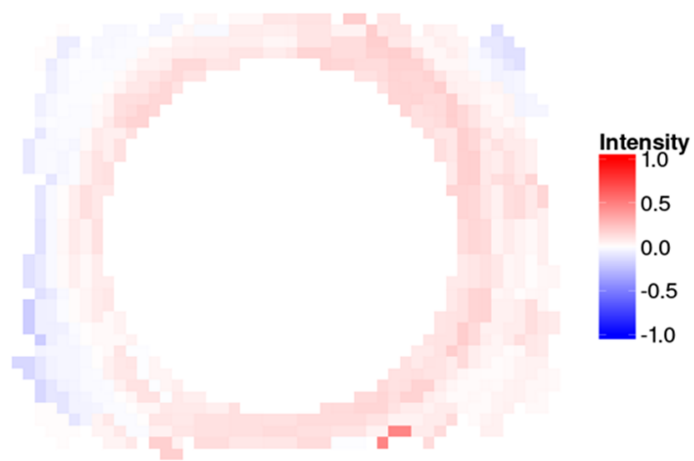
**C** Filling Times after AIT







**A** Avg Intensity Change



**B** Avg Flow Rate Change

