

# Ocular Spot Fluorometer Equipped With a Lock-In Amplifier for Measurement of Aqueous Flare

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**Received:** 30 October 2017

**Accepted:** 25 September 2018

**Published:** 21 December 2018

**Keywords:** blood–aqueous barrier; aqueous flare; light scatter; SUN grading; uveitis; ocular fluorometer; lock-in amplifier

**Citation:** Sudhir RR, Murthy PP, Tadepalli S, Murugan S, Padmanabhan P, Krishnamurthy A, Dickinson SL, Karthikeyan R, Kompella UB, Srinivas SP. Ocular spot fluorometer equipped with a lock-in amplifier for measurement of aqueous flare. *Trans Vis Sci Tech.* 2018;7(6):32, <https://doi.org/10.1167/tvst.7.6.32> Copyright 2018 The Authors

**Purpose:** To evaluate a custom-made ocular fluorometer for detection of intensity of light scatter (ILS) from the anterior chamber (A/C) as an objective measure of aqueous flare.

**Methods:** The fluorometer, equipped with a lock-in amplifier, was employed in the scatter mode to detect ILS from A/C. Measurements were performed with two illumination slit widths of 0.5 and 0.25 mm. The axial resolution at these slit widths were 80 and 200  $\mu\text{m}$ , respectively. Healthy and pseudophakic eyes, with grade 0 Standardization of Uveitis Nomenclature (SUN) score, were employed as control subjects. ILS was also recorded in a cohort of patients who had undergone phacoemulsification and showed grades 1+ or 2+ on postoperative days 1 and 4.

**Results:** The inter- and intraobserver variabilities in the measurement of ILS were not significant. In cataract patients, ILS was significantly higher on postoperative day 1 relative to healthy eyes. By day 4, ILS decreased significantly and was only marginally different from ILS in quiet pseudophakic eyes or healthy eyes. Eyes with higher SUN scores showed proportionately increased ILS. The receiver-operator characteristic analysis indicated no advantage in using the smaller slit width in discriminating ILS at different SUN scores although it provided higher axial resolution.

**Conclusions:** The lock-in-based spot fluorometer is reliable for measurement of ILS with high precision and accuracy. The measured ILS correlates linearly with SUN scores and can be used to provide a higher granularity for recording aqueous flare.

**Translational Relevance:** The instrument can be used in the clinical management of uveitis and drug development toward uveitis.

## Introduction

The breakdown of the blood–aqueous barrier (BAB) and the appearance of inflammatory cells in the anterior chamber (A/C) are the hallmarks of uveitis.<sup>1–3</sup> The collapse of BAB results in the release of serum proteins into A/C. The inflammatory cells and proteins scatter light (i.e., Tyndall effect) giving rise to aqueous flare.<sup>1,3</sup> One conventional approach to scoring the severity of uveitis is based on grading

the intensity of aqueous flare using slit-lamp biomicroscopy as per Standard Uveitis Nomenclature (SUN) scoring system.<sup>4</sup> According to SUN classification, the aqueous flare is graded 0 in the absence of any notable flare, 1+ for faint flare, 2+ for moderate flare (iris and lens details clear), 3+ for marked flare (iris and lens details are hazy), and 4+ for intense flare (fibrin in the aqueous humor).<sup>4</sup> The scoring system, albeit subjective, is frequently employed in the clinical management of uveitis.<sup>1,3–5</sup> The subjective nature of

SUN scoring leads to significant intra- and interobserver variability. Moreover, the grading does not offer sufficient granularity (i.e., subdivisions between 0 to 1+, 1+ to 2+, and 2+ to 3+) that would be essential for evaluation of the efficacy of drugs or modalities of drug delivery toward the treatment of uveitis. An enhancement in the granularity of the grading would also be helpful in early detection of recurrence.

As an alternative to SUN classification, aqueous flare has also been characterized by intensity of light scatter (ILS) measured from a focal point in A/C. Such instruments that measure ILS as a continuous index of aqueous flare, referred to as ocular flare meters, have been developed.<sup>3,6</sup> In this study, we have evaluated the use of a custom-made confocal spot fluorometer for measurement of ILS and correlated the measurements with SUN grading of aqueous flare. The unique aspect of our instrument is that it is equipped with a lock-in amplifier for the detection of ILS. The lock-in amplifier is a technology for detection of weak signals in a noisy background. Thus, its inclusion rejects contribution of ambient light and electronic noise. This can improve the precision, sensitivity, and dynamic range of the instrument. Also, the spot fluorometer being employed has confocal optics, and therefore, ILS measurements can be made precisely from a focal point in A/C without being confounded by light scatter from the cornea, iris, and lens. The results show that ILS measurements, based on the lock-in approach, are unaffected by intra- and interobserver variabilities and are able to discriminate SUN scores similar to other automated aqueous flare meters. In fact, our configuration for measurement of ILS, similar to other aqueous flare meters, also provides additional granularity for quantifying smaller increments in the intraocular inflammation with adequate statistical power.

## Methods

### Subjects

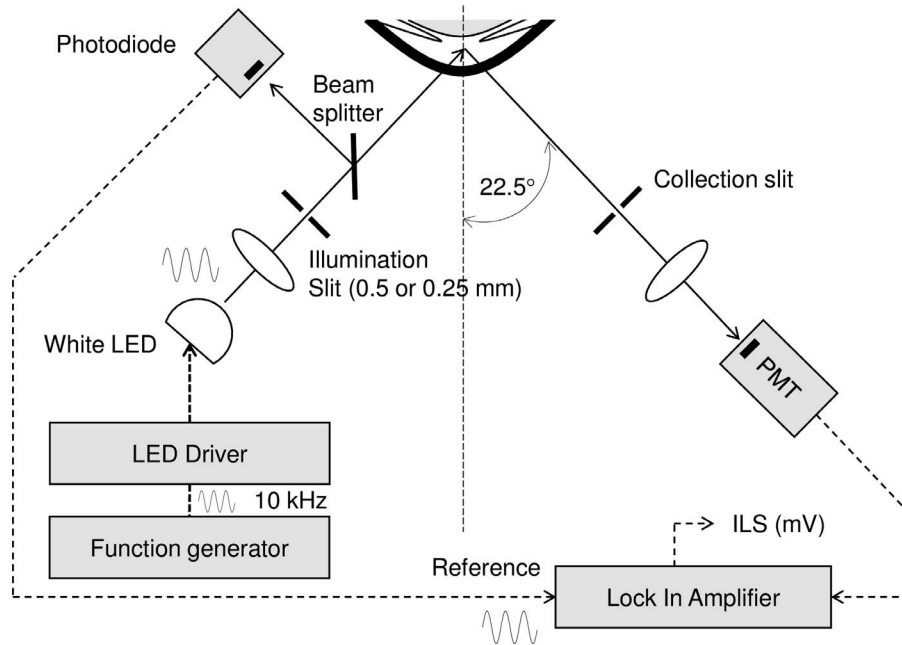
Healthy subjects, free from any ocular pathology ( $n = 50$ ; age:  $29 \pm 4$  years; 26 males and 24 females), were recruited as a control group. We also recruited subjects who had undergone uncomplicated phacemulsification more than 3 months ago as a second control group ( $n = 42$ ; age:  $64 \pm 7$  years; 16 males and 26 females).

As the study group, we recruited patients who had undergone cataract surgery (uncomplicated phacemulsification with posterior chamber-intraocular lens PC-IOL implantation;  $n = 94$ ; age:  $67 \pm 9$  years; 36 males and 58 females) on postoperative days 1 and 4. Patients with any preoperative corneal edema/scarring or showing anomalies in A/C or the vitreous cavity were excluded. Patients who had postoperative corneal edema were also excluded. SUN grading for all patients was performed by one ophthalmologist (RRS).

The postoperative treatment included topical prednisolone acetate (1% eye drops, 6 times a day; weekly tapering over 6 weeks). Informed consent was obtained from all subjects before measurements. The study protocol adhered to the tenets of the Declaration of Helsinki. It was also approved by the institutional review board at the eye hospital (Medical Research Foundation, Chennai, India).

### Measurement of ILS

We measured ILS from A/C using our custom-made confocal ocular spot fluorometer. The fluorometer, which is built around a typical slit lamp, was developed to assess tear dynamics, epithelial permeability, endothelial permeability, and aqueous humor dynamics using fluorescein.<sup>7</sup> The instrument can measure fluorescence from any desired spot in the anterior segment of the human eye. The illumination source is a white light-emitting diode (LED; 10 W). The scattered light is collected through another slit (referred to as the collection slit) held confocal to the illumination slit and positioned at the imaging port of the slit lamp. Further, the scattered light is detected by an analog photomultiplier tube (PMT; 928HA; Hamamatsu Photonics Inc., Bridgewater, NJ) (Fig. 1). To reject the interference from ambient light and to suppress the electronic noise/drifts, the current output of the PMT was further amplified by a lock-in amplifier (Model 7260, bandwidth  $\sim 250$  kHz; AMETEK Advanced Measurement Technology Inc., Oak Ridge, TN). This approach to the detection and amplification of the scattered light requires intensity modulation of the LED. As shown in Figure 1, the output (sine wave; 10 kHz) of a function generator (SR345; Stanford Research, Sunnyvale, CA) was fed to a linear amplifier (20 W at a bandwidth of 120 kHz; custom-made), which in turn, modulated the LED intensity as a sine wave. The output of a photodiode (DET10A; Thorlabs, Inc., Newton, NJ), which sampled the illumination beam, formed the reference



**Figure 1.** Configuration of the spot fluorometer for ILS measurements. Sine output from the function generator was connected to the LED driver. White modulated output was sampled by the beam splitter and led to a Si photodiode (DET10A; Thorlabs). The output of the photodiode is used as the reference to the lock-in amplifier, which was also fed with the current output of the photomultiplier. The collection slit was positioned at the imaging port so that it is confocal to illumination slit of the slit lamp. The lock-in was controlled by a desktop PC. ILS was obtained by focusing the illumination slit in the A/C at the center of the pupil.

input to the lock-in amplifier. The output time constant of the lock-in amplifier was set to 200 msec throughout the study. For measurement of ILS, two illumination-slit widths (0.5 and 0.25 mm) were employed (Fig. 2). The height of the illumination slit, however, was fixed at 4 mm. Collection slit was also of 4-mm height, but its width was adjusted to be close to that of 0.5-mm illumination slit. ILS output in millivolts from the lock-in amplifier was recorded on a PC at approximately 100 Hz via a GPIB/IEEE488 interface. The samples were averaged further to minimize the noise. Specifically, after focusing the illumination spot in A/C at the center of the pupil, sampling was begun with a click of a handheld button for 1 to 2 seconds. For each click, at least 15 to 20 samples were collected and averaged to obtain an ILS sample. In most acquisitions, 10 to 15 samples were acquired over approximately 15 seconds from each eye at a given sampling instance. All measurements of ILS were performed without dilating the pupil in a semidark room. During the measurements, subjects were asked to hold the eye open during the measurements. Furthermore, motion artifacts did not contribute much to the error in the measurements because the focal volume of the illumination beam is much smaller than the volume

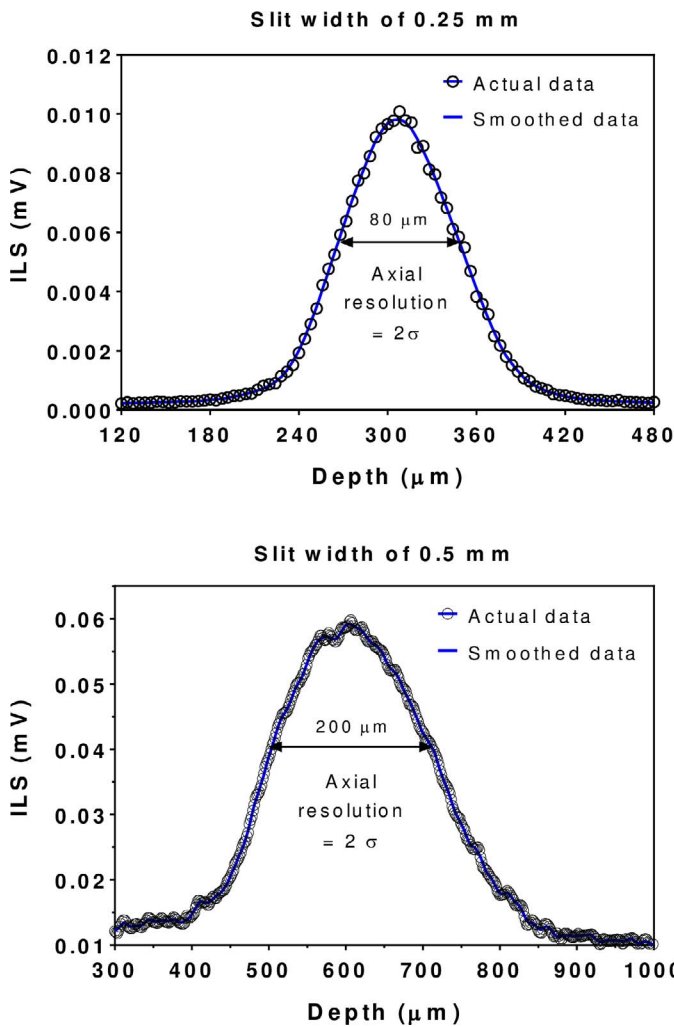
of the anterior chamber. Occasionally, we had to remove outliers because of interference from blinking. Such data could be easily identified by the high standard deviation in the measured ILS. Overall, we may have removed of 1 to 2 data points very occasionally out of 10 to 14 data points observed during any instance of ILS measurements.

### Linearity of the ILS

To assess the linearity of ILS measurements, titration experiments were performed with bovine serum albumin dissolved in 0.9% saline (0–5 mg/mL) at room temperature.<sup>8,9</sup> The freshly prepared solutions were held in a glass test tube of more than 1-cm diameter, and ILS measurements were repeated three times, with each recording having an average of at least 10 measurements with 0.5-mm slit width. The background ILS (obtained with saline in the test tube) was subtracted and plotted to assess the linearity (Fig. 3).

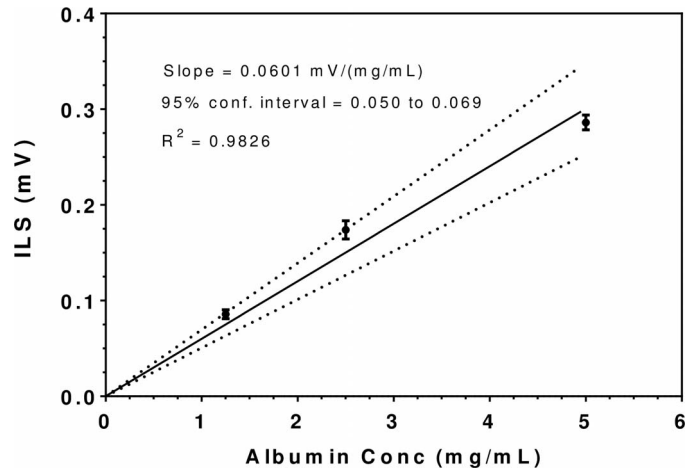
### Data Analysis

Statistical analyses and plotting were performed using SAS for Windows (v 9.4; SAS Institute, Cary, NC) and GraphPad 5.0 (La Jolla, CA). In all tests, *P*



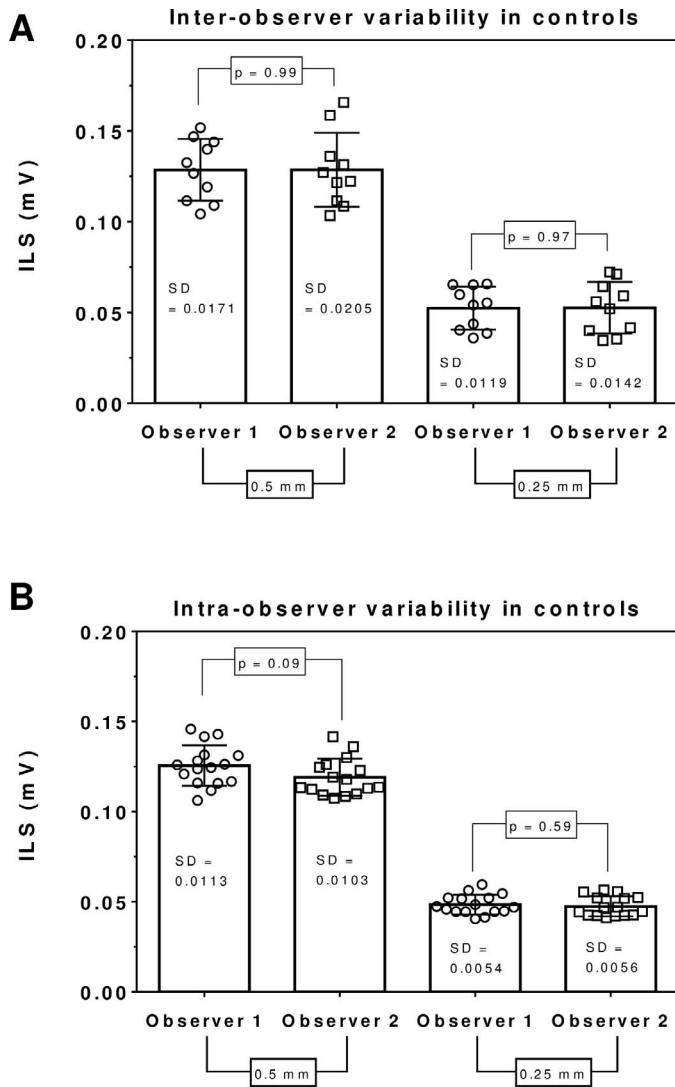
**Figure 2.** Axial resolution of the fluorometer. ILS recorded while scanning the image of the illumination slit on white paper. (A) Slit width = 0.25 mm. (B) Slit width = 0.5 mm. The responses are Gaussian, and accordingly, the axial resolution is calculated as  $2\sigma$ .

values less than 0.05 were considered statistically significant. ILS for the control and experimental groups are reported as mean  $\pm$  standard deviation in millivolts as obtained from the lock-in amplifier. Unpaired *t*-tests (two-tailed) were performed, at both slit widths, to analyze the difference in ILS between two observers, to measure interobserver variability (Fig. 4A). For intraobserver variability, each observer recorded eight to 10 measurements in the same eye, and independent sample *t*-tests were used to compare the means between two observers at both slit widths, with standard deviation used to describe variability (Fig. 4B). A Kruskal-Wallis test was used to determine the statistical significance



**Figure 3.** Linearity of the intensity of light scatter assessed with albumin. ILS was recorded with bovine serum albumin dissolved in 0.9% saline at room temperature. Freshly prepared solutions were held in a glass test tube of 1 cm, and ILS was recorded 3 $\times$ . The raw data for the three recordings after subtraction of the background are given in the *inset*. A highly significant correlation ( $r^2 = 0.9826$ ,  $P < 0.0003$ ) can be noted between the albumin concentration and ILS. The *dotted lines* show the 95% confidence band around the best-fit linear profile.

comparing ILS at different SUN grades, for each slit width, and a regression analysis evaluated the relationship of ILS on increasing SUN scores (Fig. 5; Table 2). A series of unpaired *t*-tests were used to compare ILS in patients on postoperative days 1 and 4, as well as with healthy and PC-IOL patients (Figs. 6A, 6B). Wilcoxon signed rank tests for paired data were applied to analyze the longitudinal change in ILS readings between postoperative days 1 and 4, for each slit width (Fig. 7). Logistic regression models and receiver operating characteristic (ROC) analyses were performed to identify the slit size that provides maximum sensitivity and maximum specificity in discriminating SUN scores (grade 0, 1+, and 2+) from ILS. The diagnostic accuracy of ILS in predicting qualitative SUN scores was measured with the area under the curve (AUC; determined with 95% confidence interval) and cut-off values of ROC curves (Fig. 8). The interaction term between ILS and slit size in the logistic regression models provided a significance test of whether ILS is more or less predictive of SUN scores between the two slit widths. Power analysis was performed using G\*Power (Version: 3.1.9.2; downloaded from <http://www.gpower.hhu.de/>) to determine the number of patients needed to detect a significant decrease in the mean ILS between two levels of aqueous flares with a finer



**Figure 4.** Inter- and intraobserver variability in the measurements. ILS was measured in healthy eyes because the expected magnitude of light scatter would be minimal and hence would provide a conservative measure of the S/N ratio. All measurements were performed with two illumination slit sizes of 0.5 and 0.25 mm. (A) Interobserver variability between two observers measured with the same cohort of healthy eyes (10 subjects). The interobserver variability is not significant at either of the slit settings. (B) Intraobserver variability measured for two observers. Both observers recorded more than 10 ILS measurements over a period of 30 minutes on one subject. The intraobserver variability is also not significant at either of the slit settings.

granularity than the current subjective SUN scores. The signal to noise ratio (S/N ratio) of the measured ILS signal (i.e., the ratio of mean ILS to the standard deviation of ILS) was also calculated to assess the effect of instrument noise (Table 1).

**Table 1.** Signa-to-Noise Ratio in ILS Measurements

	0.25 mm	0.5 mm	Number of Eyes
Grade 0	30.39	58.07	$n = 45$
Grade 1+	31.25	67.78	$n = 27$
Grade 2+	32.39	75.16	$n = 26$

## Results

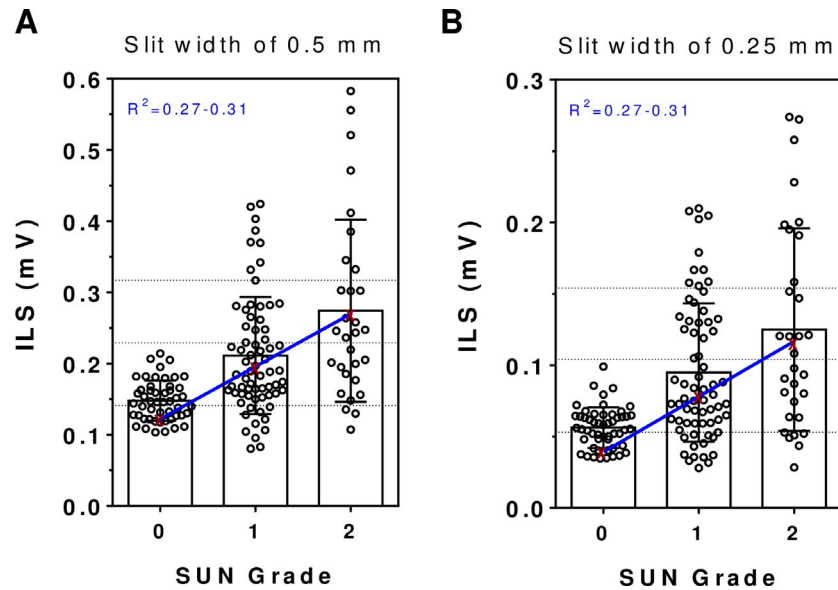
### Axial Resolution and Linearity of the Fluorometer in the Scatter Mode

The aqueous flare refers to light scatter in A/C (i.e., modulation of ILS). Therefore, a high axial resolution of the instrument is essential to minimize contributions of any scattered light from the cornea, iris, and lens. As a measure of the axial resolution of the fluorometer, we assessed changes in ILS from a slit image focused on a piece of paper with a matte finish. The width was illumination slit set at 0.25 or 0.5 mm while the slit height was maintained at 4 mm. Typical depth versus scatter intensity profiles are shown in Figure 2. The scanning was performed along the axis dividing the half-angle between the illumination and collection beams. For a Gaussian point spread function, the axial resolution is defined as the depth equal to  $2\sigma$  of the point spread function.<sup>10</sup> It is approximately 80 and 200  $\mu\text{m}$  for 0.25- and 0.5-mm illumination slit widths, respectively. These values are well below the depth of A/C in humans ( $\sim 3$  mm). Hence, data in Figure 2 suggest that our instrument is suitable for collecting ILS exclusively from A/C. In other words, the axial resolution is sufficiently high for avoiding light scatter from cornea, iris, and lens. Although both illumination-slit widths seem to be adequate, the decision would depend on the S/N ratio that we would encounter in the measurement of ILS in human subjects.

To assess the linearity of the ILS measurements, we measured ILS from albumin solutions held in a test tube at the chin rest. As shown in Figure 3, the measured ILS shows a linear correlation with albumin (0–5 mg/mL). This approach to assessing the linearity of our instrument is similar to previous reports with commercial flare meters.<sup>2,3,8,9</sup>

### Statistical Variability of ILS Measurements in Healthy Eyes

In this series of experiments, we have evaluated the S/N ratio in ILS measurements as well as determined inter- and intraobserver variabilities. As



**Figure 5.** ILS versus SUN grading of aqueous flare. ILS measured with two different slit widths in healthy eyes and patients who had undergone phacoemulsification. The SUN scores varied from 0, 1+, and 2+. The *straight line* shows a linear correlation between ILS and SUN scores ( $R^2 \sim 0.28$  for both 0.5- and 0.25-mm slit widths). The difference between ILS at different SUN grades is also statistically significant ( $P < 0.001$ ; also see [Table 2](#)).

shown in [Table 1](#), the S/N ratio measured at grade 0 (from control subjects) was approximately 30, and approximately 58 at 0.25- and 0.5-mm slit widths, respectively. The corresponding values increased slightly at higher SUN grades ([Table 1](#)). It is also evident that at all SUN grades, the S/N ratio is nearly 2-fold higher at 0.5 mm compared with the value at 0.25-mm slit width. Based on the S/N ratio alone, 0.5 mm is much better for ILS measurements, given that both slit widths are suitable based on axial resolution ([Fig. 2](#)).

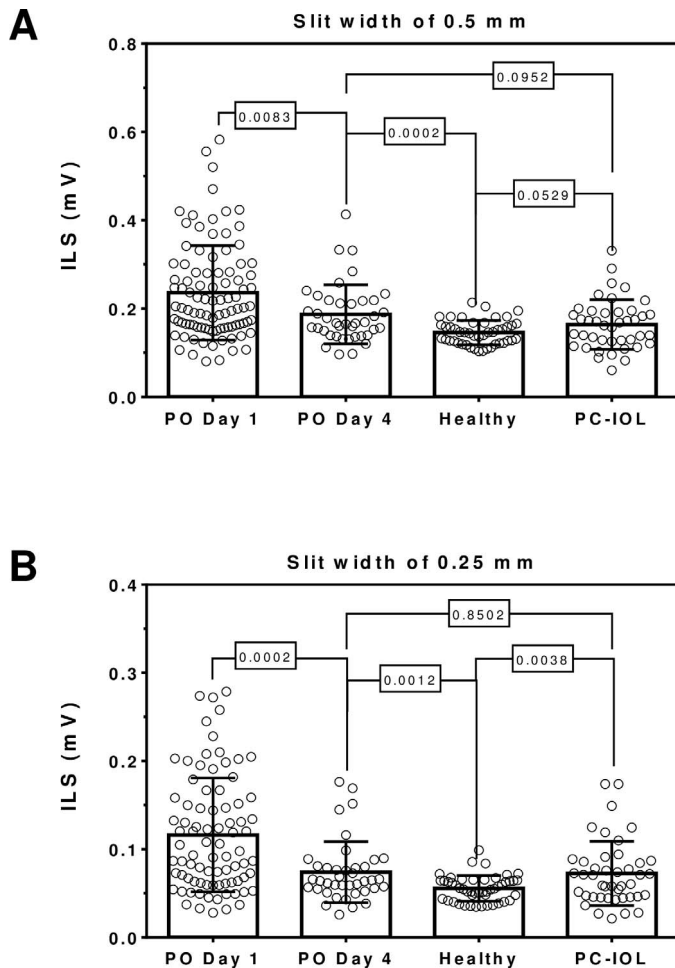
To assess the interobserver variability, two observers measured ILS from A/C using the two slit sizes (0.5 and 0.25 mm) in a cohort of control subjects (i.e., SUN grade 0). Mean ILS obtained by the two observers were identical at  $0.1286 \pm 0.0170$  and  $0.1286 \pm 0.0205$  ( $n = 10$  eyes of 10 subjects) for slit width of 0.5 mm ( $P = 0.99$ ). The corresponding values

at 0.25-mm slit width were  $0.0524 \pm 0.0119$  and  $0.0526 \pm 0.0142$  ( $n = 10$  eyes of 10 subjects), respectively ( $P = 0.97$ ) ([Fig. 4A](#)). Accordingly, there is no discernible interobserver variability in the measurements, highlighting that the measurements are not subjective at either of the two slit widths ( $P > 0.9$ ). We next assessed the intraobserver variability for the same two examiners on one control subject at the two slit widths. [Figure 4B](#) shows that the intraobserver variability is also negligible as highlighted by the small values of the standard deviation and nonsignificant differences of the means for slit width 0.5 ( $P = 0.09$ ) and 0.25 mm ( $P = 0.59$ ). Higher precision in the measurement is also suggested by the small standard deviation of ILS at the two slit widths. Overall, the data in [Figure 4](#) suggest that ILS can be measured quantitatively without significant inter- and intraobserver variabilities.

**Table 2.** Variation of ILS for Different SUN Scores at 0.5- and 0.25-mm Slit Widths

SUN Grading	ILS mV (Mean $\pm$ SD)		No. of Eyes ( $n$ )	
	0.5 mm	0.25 mm	0.5 mm	0.25 mm
Grade 0	$0.1478 \pm 0.0283$	$0.0562 \pm 0.0143$	50	52
Grade 1	$0.2112 \pm 0.0822$	$0.0948 \pm 0.0485$	68	68
Grade 2	$0.2741 \pm 0.1278$	$0.1249 \pm 0.0710$	30	31

Kruskal-Wallis tests showed ILS to be significantly different between SUN grades (i.e., 0 vs. 1+ and 1+ vs 2+) for both slit widths ( $P < 0.001$ ).



**Figure 6.** ILS in patients after cataract surgery. (A) Measurements with 0.5-mm slit width: ILS on day 1 is significantly higher compared with healthy eyes ( $P < 0.001$ ) and quiet pseudophakic eyes (PC-IOL;  $P < 0.001$ ). ILS reduced significantly by day 4 ( $P = 0.0083$ ). Moreover, ILS on day 4 is comparable to healthy controls ( $P = 0.0002$ ) and quiet PC-IOL ( $P = 0.0952$ ) indicating complete recovery from intraocular inflammation. (B) Measurements with 0.25-mm slit width: ILS on day 1 is significantly higher compared with healthy eyes ( $P < 0.001$ ) and quiet pseudophakic eyes (PC-IOL;  $P < 0.001$ ). ILS reduced significantly by day 4 ( $P = 0.0002$ ). Moreover, ILS on day 4 is comparable to healthy controls ( $P = 0.0012$ ) and quiet PC-IOL ( $P = 0.8502$ ) indicating complete recovery from intraocular inflammation.

## ILS After Cataract Surgery

To evaluate the suitability of the instrument in discriminating longitudinal changes in the aqueous flare, we chose to assess intraocular inflammation in patients who had undergone uncomplicated phacoemulsification. Specifically, we measured ILS on the first and fourth postoperative days. Contemporaneously, the intraocular inflammation in the eyes was also graded according to SUN classification by the

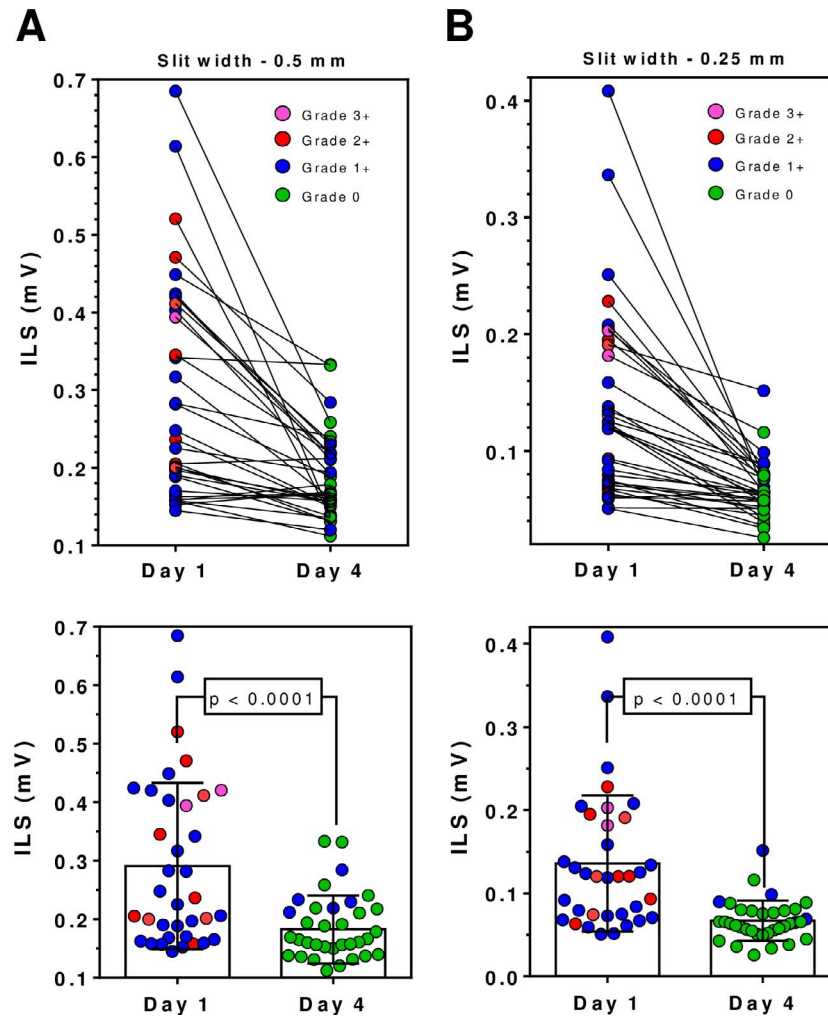
surgeon who performed the phacoemulsification (RRS). The observers recording ILS were blinded of the SUN scores of all patients.

In the cohort of patients who had undergone phacoemulsification, the SUN scores were either 1+ or 2+ concomitant with changes in ILS during the 2 days of observation (postoperative days 1 and 4). None of the subjects showed a continued increase in the aqueous flare because all patients had received prophylactic therapy. The data of all subjects have been analyzed in the following two ways: (1) ILS versus SUN scores (Fig. 5), and (2) longitudinal progression of ILS between the 2 postoperative days (Fig. 6—unpaired analysis and Fig. 7—paired analysis).

Figure 5 and Table 2 show the variation of ILS for different SUN scores. Kruskal-Wallis tests for the two slit widths showed ILS to be significantly different between SUN grades (i.e., 0 vs. 1+ and 1+ vs. 2+). A regression analysis between ILS and SUN scores also indicated a linear correlation, although with  $R^2 = 0.22$  for 0.5-mm and  $R^2 = 0.36$  for 0.25-mm slit widths.

Not all subjects in Figure 5 could be analyzed for the longitudinal progression of ILS. As shown in Figure 6A, mean ILS ( $0.1869 \pm 0.0664$ ;  $n = 40$  eyes) on postoperative day 4 is less than postoperative day 1 ( $0.2359 \pm 0.1070$ ;  $n = 94$  eyes) at 0.5-mm slit width ( $P = 0.0083$ ). ILS on day 4 is also statistically different from ILS in healthy eyes ( $P = 0.0002$ ) and is only marginally different from quiet pseudophakic eyes with PC-IOL ( $P = 0.0952$ ). We also note that ILS between PC-IOL and healthy eyes is insignificant ( $P = 0.0529$ ). Figure 6B is similar to Figure 6A but it provides a summary of ILS at 0.25-mm slit width, which offers a higher axial resolution to ILS measurements (Fig. 2). On day 1, ILS was  $0.1161 \pm 0.0646$  ( $n = 83$  eyes) and by day 4, it decreased to  $0.0740 \pm 0.0345$  ( $n = 40$  eyes), which was statistically significant ( $P = 0.0002$ ). This observation is similar to that with a 0.5-mm slit width. ILS on day 4 was significantly different from ILS in healthy patients ( $P = 0.0012$ ) but not when compared with pseudophakic eyes ( $P = 0.8502$ ). Moreover, ILS in healthy is distinctly different from PC-IOL, indicating a slight contribution of the IOL in and of itself to ILS.

Figure 7 shows a paired analysis of longitudinal data extracted from Figure 6. Consistent with the topical treatment following the surgery, the ILS decreased significantly ( $P < 0.0001$ ) in concordance with the decrease in SUN grade from 2+ to 0 (Figs. 7A, 7B). These data show that the ILS measurements are sensitive to changes in the severity of intraocular



**Figure 7.** ILS on postoperative days 1 and 4. Part of the data in Figure 6 are replotted to show changes in ILS and the corresponding SUN grades from day 1 to day 4. (A) ILS measured with 0.5-mm slit width on postoperative day 4 is significantly reduced and is also reflected by reduced SUN score ( $P < 0.0001$ ). (B) Similar to (A), but ILS measurements were performed with a slit width of 0.25 mm. ILS on postoperative day 4 is significantly reduced along with a reduction in SUN score ( $P < 0.0001$ ).

inflammation. We also measured ILS in a small cohort of patients with anterior uveitis who had significant inflammation as indicated by their SUN grades. The data, shown in Table 3, clearly show that ILS is high in patients scored with higher SUN grades.

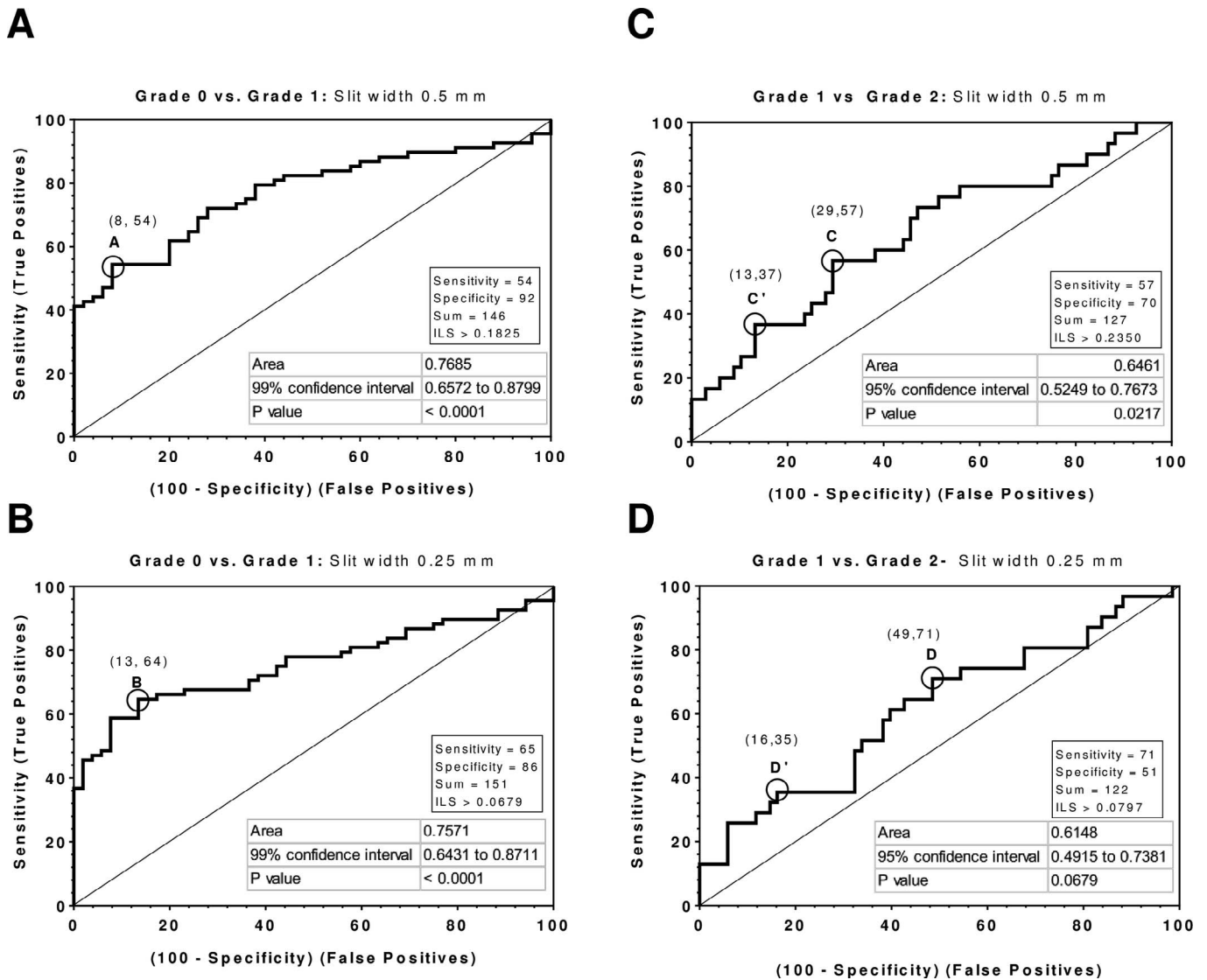
### Effects of the Illumination Slit Width in Discriminating ILS for Different SUN Scores

The measurement of ILS can be accomplished at a higher S/N ratio by widening the slit widths (Table 1). However, such a maneuver reduces the axial resolution, which increases out of focus contributions from cornea, iris, or lens to the measured ILS. This can be expected to confound the correlation between ILS and SUN scores, and thereby affecting the ILS cut-off

values for different SUN scores. Therefore, the choice of slit widths must be optimized so that the measured ILS discriminates SUN scores with maximum sensitivity and specificity. For this purpose, we constructed ROC curves at the two slit widths (0.25 and 0.5 mm) for (1) ILS of 0-grade flare as control and ILS of 1+ as an experimental variable, and (2) ILS of grade 1+ flare as control and ILS of 2+ grade as an experimental variable.

Figure 8 shows the ROC curves along with associated outputs of the estimates, such as AUC and  $P$  values. These data are presented in insets of the respective ROC curves. A high value of AUC indicates greater sensitivity in detecting ILS with the best possible specificity in discriminating two neighboring SUN scores.





**Figure 8.** Influence of the illumination slit width—analysis by ROC curves. The AUCs (given in tables provided as insets) for SUN scores 1+ at slit widths of 0.5 and 0.25 mm are 0.7685 and 0.7571, respectively. A small difference is also noted at SUN score of 2+ (0.6461 vs. 0.6148 for 0.5 and 0.25 mm, respectively). For further analysis of the influence of the slit widths, we chose the cut-off points as coordinate pairs representing the maximum sensitivity and maximum specificity of ILS. These are shown by *unfilled circles* along with the numerical values of the coordinate pair. Further, the number below a cut-off point is the sum of the coordinates. The numbers, highlighted in square boxes, are ILS at the cut-off points. At both 0.5- and 0.25-mm slit widths, the sum of the maximum sensitivity and specificity are roughly the same.

**Table 3.** ILS in Uveitis Patients With Different SUN Grades

Patient #	SUN Grading	ILS With 0.5 mm Mean $\pm$ SD, in mV	ILS at 0.25 mm Mean $\pm$ SD, in mV
1	3+	0.3209 $\pm$ 0.0455	0.1737 $\pm$ 0.0226
2	3+	0.5023 $\pm$ 0.0382	0.1567 $\pm$ 0.0125
3	2+	0.3145 $\pm$ 0.0131	0.1112 $\pm$ 0.0060
4	2+	0.3699 $\pm$ 0.0422	0.1139 $\pm$ 0.0248
5	1+	0.1970 $\pm$ 0.0089	0.0835 $\pm$ 0.0083

ROC curves in [Figures 8A and 8B](#) show that AUC at 0.5-mm slit width (0.7685) is only marginally different from AUC at 0.25-mm slit width (0.7571) indicating that both slit widths would provide a similar statistical advantage in assigning measured ILS between the SUN grades 0 and 1+. Likewise, [Figures 8C and 8D](#) show that AUCs for both the slit widths are 0.6461 and 0.6148 for SUN grades 1+ and 2+. Logistic regression models of grade (0 vs. 1+, or 1+ vs. 2+) showed a nonsignificant interaction between slit width and ILS, indicating that the discrimination ability of ILS on SUN grade was not significantly different between the two slit widths. These assessments imply that any of the two slit widths would be optimal to distinguish whether a measured value of ILS is indicative of grade 1+ or grade 2+ scores. It is also evident from the different ROC curves ([Figs. 8C, 8D](#)) that specificity values (70% at 0.5-mm slit width and 57% at 0.25-mm slit width) are too small for assigning ILS between the SUN scores of 1+ and 2+. This is consistent with the high variability in the measured ILS at grade 1+ and grade 2+ SUN scores ([Fig. 5](#)). Overall, ROC curves based on the raw data suggest no specific advantage in selecting 0.25-mm slit width over 0.5-mm slit width for ILS measurements.

## Discussion

The magnitude of aqueous flare is a predictor of the severity of uveitis<sup>11</sup> and intraocular inflammation following cataract surgery.<sup>12–16</sup> An increase in aqueous flare in response to prostaglandins has also been reported in rabbits,<sup>17–19</sup> although not in humans. The aqueous flare is also elevated during diabetes,<sup>20,21</sup> retinal detachment,<sup>22–25</sup> and retinitis pigmentosa.<sup>26–28</sup> In order to monitor the progression and recurrence of the intraocular inflammation, many investigators have measured ILS as a continuous index of aqueous flare using laser flare meters in addition to the subjective SUN classification.<sup>4</sup> Because our newly constructed confocal spot fluorometer is specifically designed to reject ambient light and electronic noise/drifts, we sought to make use of the fluorometer to measure ILS for characterizing the aqueous flare quantitatively. The confocal optics of our instrument motivated us further to undertake the measurements because ILS can be collected exclusively from A/C. The main finding based on our measurements with cohorts of healthy eyes and postcataract surgery patients is that the ILS measured with our instrument varies linearly with increase in the severity of the

inflammation as adjudged by SUN scores.<sup>3</sup> Secondly, we also found the measurements to be independent of intra- and interobserver variabilities, which is also the case in laser flaremeters.<sup>3</sup> Finally, the linearity of the instrument ([Fig. 3](#)) and S/N ratio in the measurement of ILS ([Table 1](#)), as well as intraobserver variability ([Fig. 4B](#)), suggest that grading of the intraocular inflammation can be resolved with a higher granularity compared with SUN scores.

Although the above observations are not different from those already reported with commercial laser flare meters, the two features of our fluorometer are noteworthy. First, we have registered a high axial resolution, relative to the depth of A/C, with our instrument as shown in [Figure 2](#). Secondly, we have measured ILS by synchronous detection via inclusion of a lock-in system for detection and amplification of the light scatter ([Fig. 1](#)). Hence, ILS measurements are unaffected by ambient light and electronic noise outside of the reference frequency. This is especially true because most digital lock-in amplifiers reject noise in excess of 80 dB outside of the reference frequency. Our choice of 10 kHz for modulation of the LED ([Fig. 1](#)) is significantly far from potential contributions by flickering light sources around the instrument that may be present in a clinical setting. Thus, only the scatter signal, which is at the modulation frequency of the illumination beam, would be amplified (i.e., amplification of the scatter signal synchronous with reference frequency at 10 kHz). In other words, ambient light (usually at low frequencies) and electronic noise (potentially at high frequencies) are effectively rejected. This noise reduction ensures that the changes in ILS are affected exclusively by changes in aqueous flare, leading to an increase in sensitivity. Thus, our fluorometer, similar to other laser flare meters already in the market, can be expected to delineate smaller incremental changes in intraocular inflammation with precision. In other words, we can detect a small change in the intraocular inflammation with a fewer number of subjects at the desired level of statistical power and  $\alpha$  error.

In the small range of SUN scores in our study (0, 1+, and 2+), ILS measurements not only showed precision but also exhibited a linear correlation with SUN grades ([Fig. 5](#) and [Table 2](#)). The variability in ILS for a given SUN grade ([Fig. 5](#) and [Table 2](#)) can be attributed to the subjectivity in SUN scoring. The problem further exacerbated by the poor granularity of SUN classification. This claim is based on our

observation that intra- and interobserver variabilities in ILS measurements are negligible (Fig. 4) even with a cohort of healthy subjects in whom ILS is very small and hence noisy. More specifically, the standard deviation in the measurements is of the order of 0.001 mV (Fig. 4), which truly reflects the instrument + observer variability.

The effect of prophylactic therapy in our subjects is evident in Figure 6 as demonstrated by others using laser flare meter.<sup>3</sup> ILS decreased significantly on postoperative day 4 compared with day 1 correlating with the decrease in inflammation as per SUN scoring. In fact, by the postoperative day 4, most patients reached near grade 0 as shown in the longitudinal observations highlighted in Figure 7. Moreover, we note that postoperative day 4 values are indistinguishable from ILS in pseudophakic eyes (PC-IOL;  $P = 0.0952$  at 0.5-mm slit width and 0.8502 at 0.25-mm slit width). The large standard deviations in the measured ILS in Figures 5, 6, and 7 are attributable to the poor granularity of SUN grades.

In the absence of prior knowledge on the S/N ratio in ILS measurements that we would get with human subjects, we began the study with two slit widths. The larger slit width has the disadvantage of lower axial resolution, which can result in the collection of light scattered from around the focal point in A/C. The small slit width has the potential to offer high axial resolution but possibly with a reduced S/N ratio. After examining the impact of both the slit widths at different SUN grades, the larger slit width is better suited for our measurements based on the following analyses. First, referring to Table 1, it is evident that S/N ratio at 0.5-mm slit width is almost double that we note with 0.25-mm slit width for all SUN scores. We also note a slight increase in S/N ratio at both 0.5- and 0.25-mm slit widths for elevated SUN scores as well (Table 1). This increase can be attributed to the increased magnitude of ILS although it would have been reasonable to expect increased noise in the signal due to a higher flux of particulates (proteins and cells) into and out of the focal volume at higher SUN grades. Secondly, the ROC curves for different SUN scores indicate that AUCs for the two slit widths are only marginally different (Fig. 8). This observation suggests that it is acceptable to use 0.5-mm slit width to maintain higher specificity in delineating aqueous flare.

To detect a statistically significant change in mean ILS for a group of patients, between two levels of aqueous flare, we can consider a specified percent increase in inflammation over 0 grade. Based on the

standard deviation of the interobserver variability for 0.5-mm slit width (Fig. 4B), we estimate that for a 33% increase in inflammation over the grade 0 can be detected at a statistical power of 0.99 and  $\alpha$  error of 0.05 using a cohort of 16 subjects (8 as control and 8 as experimental). Likewise, we estimate that 33% increase in inflammation over the grade 1+ requires a cohort of 14 subjects (7 as control and 7 as experimental). An alternative way to assess the statistical power to discriminate a certain degree of increase/decrease in aqueous flare can be based on the standard deviation in the measured ILS. Such a power analysis for determining the sample sizes (power of the test at 0.99 for an  $\alpha$  error of 0.5) indicated that we would need cohorts of six subjects (3 as control and 3 as experimental) and four subjects (2 as control and 2 as experimental) to detect a 33% increase in aqueous flare from grade 0 and grade 1+, respectively.

The estimated sample sizes, discussed above, can be further reduced by decreasing the variability in the measured ILS. For reducing the variability in ILS, we can improve light detection (e.g., with a more sensitive photomultiplier tube), enhance collection efficiency of the scattered light, or increase the output time constant of the lock-in amplifier (from 200–500 ms). The caveat for the latter approach is the possibility of adversely increasing the motion artifacts in ILS detection. In any case, there is ample scope for further optimization of our instrument for enhanced detection sensitivity and specificity.

Overall, we have shown a simple technique for assessing aqueous flare that could be used for monitoring the efficacy of pharmacologic strategies quantitatively, and possibly, aid in early detection of relapses in uveitis patients.

## Acknowledgments

Supported, in part, by grants from the Indiana Clinical and Translational Sciences Institute funded, in part by Grant Number (Grant # UL1TR001108) from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award (SPS). Work also partially supported by Obama-Singh Initiative (2014-2018; SPS and PP).

Disclosure: **R.R. Sudhir**, None; **P.P. Murthy**, None; **S. Tadepalli**, None; **S. Murugan**, None; **P. Padmanabhan**, None; **A. Krishnamurthy**, None; **S.L.**

Dickinson, None; R. Karthikeyan, None; U.B. Kom-pella, None; S.P. Srinivas, None

\*PPM, ST, and SM contributed equally to this work.

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