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Corresponding Author:	Jordan Rossy University of Waterloo Faculty of Science Waterloo, Ontario CANADA
Corresponding Author's Institution:	University of Waterloo Faculty of Science
Corresponding Author E-Mail:	j2rossy@uwaterloo.ca
Order of Authors:	Jordan Rossy
	David McCanna
	Bernard Fresco
	Jacob Sivak
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TITLE:

Organ Culture System for Assessing the Toxicity of Intraocular Treatment Excipients and Pharmaceuticals

AUTHORS AND AFFILIATIONS:

Jordan Rossy[1], David J. McCanna[2], Bernard Fresco[3], Jacob G. Sivak[1]

[1]School of Optometry and Vision Science, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

[2]Centre for Ocular Research & Education, School of Optometry and Vision Science, University of Waterloo, 200 University Ave West, Waterloo, Ontario, Canada N2L 3G1

[3]Tonomed Inc, 40 St Clair Ave E., Toronto, Ontario, Canada M4T 1M9

Email addresses of co-authors:

David J. McCanna (djmccann@uwaterloo.ca)

Bernard Fresco (drfresco@eyeknowcenter.com)

Jacob G. Sivak (jsivak@uwaterloo.ca)

Corresponding author:

Jordan Rossy (j2rossy@uwaterloo.ca)

KEYWORDS:

In vitro, bovine lens, organ culture, cell culture, metabolic activity, cataract, lanosterol, benzalkonium chloride, toxicity, lens laser-scanner, optical quality

SUMMARY:

The goal of this protocol is to evaluate changes in metabolic activity and refractive function of the lens in response to experimental treatment.

ABSTRACT:

As the leading cause of blindness, cataracts are a significant burden for the tens of millions of people affected globally by this condition. Chemical exposures, among other environmental factors, are an established cause of cataracts. Ocular toxicity testing can assess whether pharmaceuticals and their components may contribute to lens damage that may lead to cataracts or aid the treatment of cataracts.

In vitro studies and *in vivo* animal testing can be used for assessing the safety of chemicals prior to clinical studies. The Draize test—the current *in vivo* standard for ocular toxicity and irritancy testing—has been criticized for lack of sensitivity and objective measurements of determining ocular toxicity. *In vitro* cell-based assays are limited as cell cultures cannot appropriately model an intact functional lens.

The method described here is a sensitive *in vitro* alternative to animal testing, designed to evaluate the response of the intact bovine lens to treatment at both the cellular activity level

and for overall refractive performance. The non-toxic reagent resazurin is metabolized in proportion to the level of cell activity. The lens laser-scanner assay measures the ability of the lens to refract incident beams of light to a single point with minimal error, directly relevant to its natural function. The method may be used to determine both acute and delayed changes in the lens, as well as the recovery of the lens from chemical or environmental exposures.

INTRODUCTION:

Affecting over 20 million people, cataracts are the most prevalent cause of blindness worldwide[1,2]. Cataracts are most commonly due to age-related changes in the lens but are also induced from trauma, genetic conditions, disease, or toxic exposures[2]. Currently, treatment involves surgical intervention to replace the lens, an expensive and invasive procedure accessible mainly to developed countries. The extensive burden of cataract has directed decades of research towards cataract prevention and the development of non-surgical treatment. In both cases, the importance of preclinical testing for toxicity, efficacy, and pharmacokinetics of ophthalmic drugs is paramount. This process of drug development relies heavily on the information provided by studies performed in animals.

The current standard for ocular toxicity testing *in vivo* is the Draize test, involving the delivery of a test compound to the conjunctival sac of a live animal. The test has been significantly criticized, particularly concerning animal ethics, subjectivity, poor repeatability, and variability[3]. Additionally, there is no component of the Draize test that directly monitors the effects of test substances on the lens. Considerable effort has been invested in developing alternative *in vitro* models[4]. However, none have been sufficiently validated to replace the Draize test[5]. Similarly, many of these models face limitations with respect to the direct application to cataracts and other complex pathologies[6]. For example, methods grading lens transparency when placed over a grid are inherently subjective[7]. Cell culture studies are reliable and highly utilized, though cell monolayer characteristics may diverge from primary tissue culture[8].

Whole lenses can be dissected from the eyes of animals and cultured to maintain their original structure and function. One assay that is useful for assessing lens function while maintaining the organ's condition is the lens laser-scanner assay involving a scanner developed at the University of Waterloo in Canada. The assay is a scanning system that uses a series of laser projections to measure the optical quality or refractive performance of the lens. Lenses are scanned in their custom two-segment culture chambers, allowing beams to pass from below through the lens (**Figure 1A**). A camera fixed inside the scanner captures the image of the laser passing through the lens at numerous points. The scanner software computes the distance behind the lens at which it intersects with a central axis (back vertex distance, BVD), producing a series of measurements that indicate how consistently the lens focuses light to a single point (**Figure 1**).

The cellular properties of the lens, such as the tight and ordered arrangement of its cells, help maintain transparency and minimize scatter so that the lens can functionally focus light[9]. This measure can be used to interpret how significantly a chemical disrupts the essential structure of the lens, such as the gradient refractive index, and how much function is compromised

because of the induced opacities. Other studies that have followed the response of cultured lenses and lens vesicles suggest that light scatter is a product of structural changes, compared to metabolic changes, and that disruptions to lens lipids and proteins may affect the refractive index and consequently increase scatter[10,11].

The lens laser-scanner can be used in conjunction with metabolic reagents in assays to determine biochemical measures of cell toxicity. Resazurin is a non-toxic chemical reagent metabolized by active cells, producing a reduced product (resorufin) with a measurable fluorescence[12]. The lens is largely devoid of organelles, except the metabolically active mitochondria concentrated within the anterior epithelium and superficial cortical fiber cells, fulfilling lens energy requirements[13,14]. Damage to the lens at the cellular level may disrupt metabolism and often precedes the onset of pathogenic structural changes and cataract[15].

The purpose of this method is to evaluate the effect of xenobiotic and environmental exposures on the lens, which may contribute to cataract development. The protocol involves two assays to evaluate the effect of a treatment using the cultured bovine lens. The advantage of this approach is that it provides both a cellular and functional evaluation of how the lens as a primary tissue responds to treatment. It is a sensitive and objective evaluation of the lens as compared to other common methods[16-18].

The model has been successfully used to evaluate the effects of various exposures, including surfactants, consumer products, alcohols, and ultraviolet radiation[17,19,20]. Changes in optical quality are consistently present in cultured lenses as a response to toxic exposure[21]. The ability of this method to maintain long-term lens culture is well-suited for monitoring the potentially delayed effect of a compound and the recovery of the lens from induced damage or cataract[22,23]. Results produced from the application of this protocol can be used to reduce dependence on animal testing in the development of ophthalmic products.

PROTOCOL:

All experimental protocols were carried out in compliance with the University of Waterloo ethics policies for research using animal tissue. The bovine eyes for the current study were abattoir-provided, obtained from non-dairy cows within a few hours of death, and were dissected immediately, a process that takes up to 8 h from obtaining the eyes. Eyes should be dissected immediately to preserve sterility and dissection quality. The culture medium is prepared to a pH of 7.4 and sterile-filtered prior to supplementation with FBS[21]. All procedures are carried out under sterile conditions, with material and equipment sources listed in the **Table of Materials**.

1. Bovine lens culture

1.1. Dissect lenses by removing extraocular muscles and tissue from the sclera, removing the posterior half of the globe and vitreous, removing the iris and ciliary body together with the lens, then cutting away the zonular attachments, with the final cut positioned such that the lens

will drop into the medium-filled culture chamber.

1.2. Culture the dissected lenses in custom chambers with a base adapted to fit the laser-scanning system, with 21 mL of sterile-filtered medium, at 37 °C and 5% CO_2. Use 3% fetal bovine serum (FBS)-supplemented culture medium with 1% penicillin-streptomycin, 9.4 g/L M-199, 0.1 g/L L-glutamine, 5.96 g/L HEPES, 2.2 g/L sodium bicarbonate, and 7 mL/L NaOH. Store unused culture medium in the refrigerator for up to 7 days.

1.3. Replace the culture medium every 1–2 days. Warm the culture medium for an hour at 37 °C prior to use. Use a suction-connected sterile Pasteur pipette to aspirate the medium from an individual culture chamber and replenish immediately with the supplemented medium.

1.4. Culture the lenses for 48 h after dissection to allow time for physical damage to manifest. Inspect and scan the lenses for optical quality according to section 4 prior to inclusion in an experiment.

NOTE: Lenses are oriented face-down in preparation for the laser-scanner assay.

2. Control procedure

2.1. Prepare a solution with the desired concentration of a solubilizing agent that is compatible with the chemical compound and culture medium.

2.2. Prepare a vehicle control solution by adding the solubilizing agent to supplemented medium. Warm the control solution for an hour at 37 °C prior to experimentation.

2.3. Use a Pasteur pipette connected to suction to aspirate the culture medium from control lens chambers. Replenish the chambers with the control solution. If required, perform this step with samples in triplicate.

NOTE: A minimum volume of 7 mL is required to completely cover the lens in the anterior face-up position. The conditions for controls in the current study were 21 mL of untreated medium control, 21 mL of a vehicle control medium, and 7 mL of phosphate-buffered saline (PBS).

2.4. Culture the lenses in control medium for 2 days, then replace with new control medium according to section 1. For short-term exposures, aspirate the control solution from the chambers and perform a rinse at least three times with unsupplemented culture medium before resuming the lens culture protocol as described in section 1.0.

2.5. Allow the lenses to acclimate in the incubator at 37 °C and 5% CO_2 for at least 3.5 h prior to the assessment of optical quality.

3. Exposure procedure

3.1. Prepare a solution with the desired concentration of a solubilizing agent that is compatible with the chemical compound and culture medium. Combine the chemical with the solubilizing agent, allowing the appropriate time for interaction if required.

NOTE: 2-Hydroxypropyl-β-cyclodextrin was used in the current study to enhance the solubility of lanosterol (0.033 g/L) in medium. Benzalkonium chloride (BAK 0.0075%) solution was prepared using PBS.

3.2. Incorporate the solubilized chemical into the culture medium. Prepare a total volume sufficient to provide 21 mL of medium to all lens samples. Warm the experimental medium for an hour at 37 °C prior to experimentation.

3.3. Use a Pasteur pipette connected to suction to aspirate the culture medium from the lens chambers. Immediately replenish the chambers with the test solution. After the exposure interval, replace with supplemented culture medium, performing a rinse first if needed. If required, perform this step with samples in triplicate.

NOTE: In the current study, test conditions were 21 mL of lanosterol-supplemented medium and 7 mL of a BAK 0.0075% solution. A minimum volume of 7 mL is required to completely cover the lens; in this case, lenses must first be oriented anterior face-up. A Pasteur pipette can be used to create a current in the culture chamber with some of the surrounding culture medium to repositions the lens.

3.4. Return the lenses to the incubator for at least 3.5 h to acclimate prior to the assessment of optical quality.

NOTE: Lenses are oriented face-down in preparation for the laser-scanner assay.

4. Optical quality assay (lens laser-scanner)

4.1. Ensure that the lenses are oriented with the anterior face down and visually level in the culture chamber, using the technique from step 3.3 to adjust the position of the lens if needed.

NOTE: This position ensures the incident beam will be reflected up through the chamber bottom and pass through the lens from the anterior to the posterior surface.

4.2. Position a lens culture chamber into the laser-scanner such that the chamber carriage pin fits into the slot in the chamber base.

4.3. Select the **lens** and **scanpoint** for which to perform a scan, right-click on **scan lens,** and wait for the scanner prompts for the beam to be found and aligned. Select well-distanced beginning and endpoints for the beam behind the lens. Ensure the central beam is aligned using the **gross** and **fine** adjustment buttons in the scanner software and adjustment knob on the scanner. When the beam is aligned, click **calibrate** and input an appropriate selection of **radial steps** and

beam separation.

NOTE: For this study using the bovine lens, the settings for **radial steps** and **beam separation** were set to **10 steps** and **0.5 mm**, respectively. The first scan that the scanner performs serves to calibrate the laser-scanner. This is required once, and all additional lens scans may be performed directly after alignment.

4.4. Once the scanner has been calibrated, click **scan**. Wait for values to be generated for BVD mean, standard deviation, and error along one axis after completion of the lens scan, and the scope of the beam has been set. Set the scope from where beams exit the lens to the desired endpoint, selecting the maximum distance behind the lens while excluding any apparent interference.

4.5. Use the generated chart to view the individual back vertex distances for each beam.

NOTE: Exclude beams passing through lens sutures. To exclude a beam, use the on-screen legend to right-click on an individual beam and select **exclude**.

4.6. Manually pivot the position of the culture chamber base inside the scanner by 90°, such that the second scan along the anterior lens surface will be perpendicular to the previous scan. Ensure the central beam is aligned as detailed in step 4.3, and then scan the lens.

5. Metabolic activity assay (resazurin)

5.1. Prepare medium without FBS. Warm the medium for an hour at 37 °C. Prepare 50 mL of 8% resazurin reagent in unsupplemented medium.

NOTE: It is sufficient to prepare 50 mL to fill all wells in a 12-well plate. The solution should be prepared sterile under dim conditions in an opaque container, as resazurin is light-sensitive.

5.2. Add 3.8 mL of 8% resazurin solution to each well within a clear-bottom, sterile 12-well plate to cover the lens.

5.3. Use sterile metal scoops to carefully lift lenses from under the posterior surface and perform a rinse using a Pasteur pipette to wash the lens with a small volume of unsupplemented culture medium. Place the lenses individually in each well.

5.4. Incubate the well plate at 37 °C and 5% CO_2 for 5 h. After the incubation, remove the lenses with metal scoops from the wells and place them in an animal waste disposal container. Transfer a 100 µL sample from each well into a sterile, clear-bottom, 96-well plate.

NOTE: Lenses may be assessed multiple times with the metabolic activity assay. In this case, return the lenses to the culture chambers with fresh medium for incubation. The metabolic activity assay is best performed once all scans are completed, as the dye may affect the BVD

measurements.

5.5. Measure the endpoint fluorescence of the 100 µL samples using a fluorescent plate reader, with the excitation and emission wavelengths set at 560 nm and 590 nm, respectively.

6. Data analysis

6.1. For the optical quality assay, calculate the average of the software-calculated values for BVD error[24] from the two scans performed at each time point. Perform this calculation for all lenses and all scanpoints.

6.2. For the metabolic activity assay, collect the endpoint relative fluorescence values generated by the plate reader. Normalize the data by setting the average of all control values at a scanpoint to 100%. Calculate the activity of each lens as a percentage of the average control value for that scanpoint.

6.3. Perform a normality test for all experiment groups. Assuming the data are normal, analyze the BVD error differences between control and experimental lenses using the two-way ANOVA. Additionally, perform Tukey's post-hoc multiple comparisons test. Perform a one-way ANOVA and Dunnett's post-hoc test for the metabolic activity data.

REPRESENTATIVE RESULTS:

Figure 2 and **Figure 3** (n = 6) demonstrate the results of a study testing the effect of chemical treatment (lanosterol) on the bovine lens. Lanosterol is a naturally occurring sterol in the lens that once showed promising results as a potential pharmaceutical intervention for cataracts[25], although this has yet to be proven[26]. The study design included a medium and vehicle control for the compound. There was no significant difference between the vehicle (2-hydroxypropyl-β-cyclodextrin) and medium control ($p > 0.05$), indicating that any potential effect in the experimental group is not likely to be due to the vehicle. There was no significant difference in BVD variability between the treatment and the control groups ($p > 0.05$). These results were consistent with the metabolic activity assay (**Figure 3**). Therefore, the treatment did not introduce significant toxicity to the cells or significantly affect the refractive performance of the lens ($p > 0.05$).

Figure 4 and **Figure 5** (n = 3) show the results of treatment with BAK on the lens. BAK is a surfactant and the most commonly used preservative in ophthalmic formulations[27]. A 10 min exposure resulted in significantly greater BVD variability in the treated lenses compared to the control at 4 days postexposure ($p < 0.05$). The treatment also produced a significant difference in lens metabolic activity ($p < 0.05$).

FIGURE AND TABLE LEGENDS:

[Place Figure 1 here]

Figure 1: Determination of back vertex distance as a measure of optical quality using laser-scanner. (**A**) A series of beams are passed through the lens while it is seated in its culture chamber along one axis. (**B**) The beams pass through the lens at specified intervals. The back vertex distance is determined for each beam, and BVD mean (in mm) and BVD error values are generated as quantitative measures of lens refractive function. This information is displayed graphically, with BVD shown on the x-axis and beam position on the y-axis. The more sharply that beams are focused to a consistent point behind the lens (**C**), the lesser the calculated BVD error value compared to lenses of poorer optical quality (**D**). Abbreviation: BVD = back vertex distance.

[Place Figure 2 here]

Figure 2: Effect of lanosterol suspension on bovine lens optical quality. Back vertex distance variability reflects the ability of the lens to refract light to a single point. The optical quality of lanosterol-treated lenses was similar to that of untreated medium and vehicle control lenses ($p > 0.05$) ($n = 6$). The data are represented as mean ± standard deviation. Abbreviation: BVD = back vertex distance.

[Place Figure 3 here]

Figure 3: Effect of lanosterol suspension on the metabolic activity of the bovine lens. Mean metabolic activity of bovine lenses, quantified by the relative fluorescence of a metabolized indicator after exposure to a vehicle-suspended lanosterol treatment ($n = 6$). The data are represented as mean ± standard deviation.

[Place Figure 4 here]

Figure 4: Effect of benzalkonium chloride on bovine lens optical quality. An exposure to BAK 0.0075% for 10 min produced gradually increasing back vertex distance variability within the treatment lenses ($n = 3$). Differences were significant between treated and medium control lenses 4 days postexposure, as well as for the treated lenses between their preexposure and postexposure scanpoints ($p < 0.05$). The data are represented as mean ± standard deviation. Abbreviations: BVD = back vertex distance; BAK = benzalkonium chloride.

[Place Figure 5 here]

Figure 5: Effect of benzalkonium chloride on the metabolic activity of the bovine lens. The endpoint of metabolic activity was measured 4 days after a 10 min exposure to BAK 0.0075% ($n = 3$). Changes in metabolic activity were significantly different from the control ($p > 0.05$). The data are represented as mean ± standard deviation. Abbreviation: BAK = benzalkonium chloride.

DISCUSSION:

The purpose of this protocol is to directly evaluate the effects of chemicals or environmental

exposures on the lens in primary tissue culture. First, lenses are dissected and scanned for optical quality. Prevention of contamination and ensuring dissection quality are critical. Lenses are scanned at periodic intervals to continuously monitor changes in refractive function with respect to the control group or preexposure condition. The metabolic activity assay represents an endpoint to determine whether the exposures have impacted cellular metabolism. These are the critical steps to determine whether a xenobiotic substance or environmental condition causes significant toxicity, potentially leading to cataracts and whether the lens may recover from this treatment.

Lenses are scanned for optical quality within their respective culture chambers. Although lenses can also be exposed to a test substance within their chambers, one of the limitations of this protocol is that lenses are sensitive to changes in osmolarity and must be continually nourished with serum and maintained within an appropriate medium. This presents a challenge for treatments with long exposure intervals or poor solubility within culture medium[28]. As the assay uses video imaging, suspensions with large amounts of insoluble particles may introduce scatter, which is not an indication of lens performance. The conditions that produced the representative data using a lanosterol suspension indicate that the protocol can tolerate certain low-level concentration suspensions. While it has been suggested previously that the cultured bovine lens can correlate with responses in the human lens[21], key differences, including but not limited to UV filtration, age-related compaction, and phospholipid content, limit the range of substances for which this protocol is appropriately used in preclinical testing[29,30].

Ocular toxicity testing necessarily involves a large battery of tests to determine a broad picture of the safety and tolerance of a compound, beginning with *in vitro* and animal testing before proceeding to clinical trials. The bovine lens assay has high throughput for the number of times a lens can be scanned as the method is non-destructive and can be performed easily within a few minutes. However, testing large numbers of lenses can have a low throughput, as dissecting a lens from an eye can be time-consuming. Use of the laser-scanner has been more broadly used to study guinea pig, fish, pig, rat, and chick lenses[31-35]. Ideally, the results of preclinical testing provide insight into the safety and potential risk in humans. While human lenses would be most useful in this respect, as human and animal lenses will inevitably differ in some cases[36], abattoir-provided lenses are useful in the balancing of available resources and ethics. This protocol represents a sensitive, reproducible, non-toxic, and objective *in vitro* method for testing both the cellular and functional conditions of the lens in response to treatment.

In comparison with the current *in vivo* standard for ocular toxicity testing, the lens laser-scanner provides a direct assessment of the effects of potentially toxic exposures on the lens. Owing to the common embryological tissue origin of the lens and cornea, as well as functional similarities such as transparency and refraction, the primary culture of the lens represents a suitable model for ocular irritancy. Preliminary validation studies of the lens laser-scanner have shown comparable results with respect to the Draize test and have even been shown to be more sensitive without inflicting any discomfort onto a live animal[18]. These results are additionally collected objectively, without the interpretation of an observer.

The lens laser-scanner assay measurements are directly relevant to the natural function of the lens *in vivo*. Moreover, unlike assays that culture the cornea or cell lines, the bovine lens maintains its refractive function using the long-term cell culture method developed, and the optical quality assay can be performed while maintaining the lens in its environment. The result is that, unlike other assays that produce a single endpoint as a result of the test itself damaging the lens, the optical quality assay can be performed repeatedly while successfully culturing the lens for up to 1000 h[24].

As the lens is largely devoid of organelles, with the exception of the anterior epithelium and superficial cortical fiber cells, these cells perform organelle functions for the entire lens[37]. It is straightforward then to understand the connection between cellular changes and the induction of lens cataracts, as observed *in vitro* and *in vivo*[15,19]. Lens metabolic activity essentially represents the activity of the anterior epithelial monolayer. While assays similar to resazurin are available, for example, tetrazolium salts including MTT, XTT, MTS, and WST, resazurin provides a non-toxic and sensitive assay highly compatible with primary lens culture. Unlike MTT, which requires the solubilization of precipitated crystals, the resazurin protocol does not involve solutions that are likely to induce lysis. Additionally, cell culture studies have implied that the resazurin endpoint is more sensitive than tetrazolium salt assays[38].

This method is designed to model the response of the lens as an ocular tissue and optical device to various chemical and environmental exposures. The two representative compounds chosen for this investigation are benzalkonium chloride, a preservative in ophthalmic solutions, and lanosterol, a sterol previously studied as part of an effort to find pharmaceutical interventions for cataracts. The results demonstrate lens stress in response to the preservative and no significant response to lanosterol. This method could be used further to study the toxicity of potential pharmaceutical treatments for cataract.

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DISCLOSURES:
The authors have no conflicts of interest to disclose.

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Figure 1

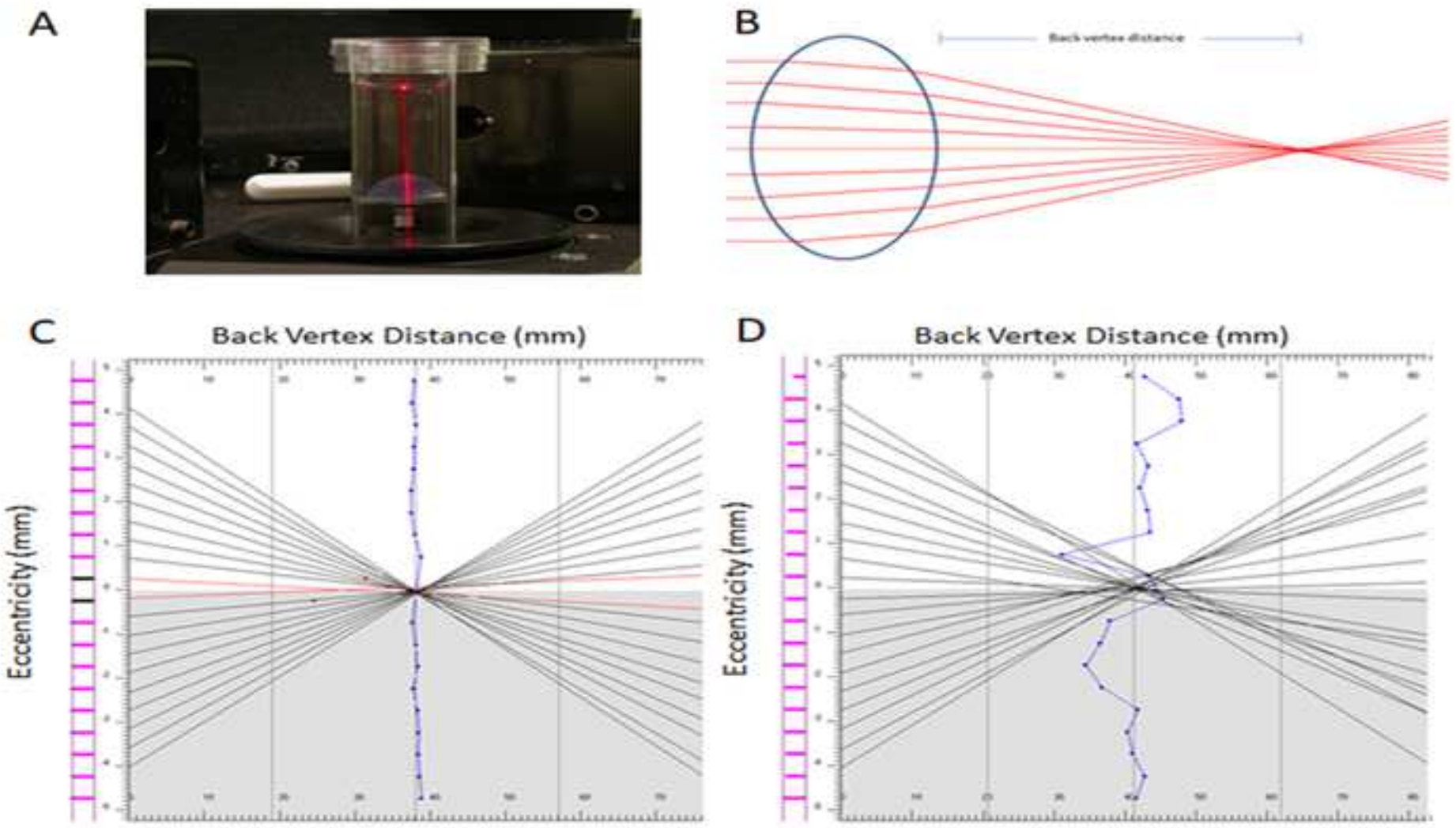

Figure 2

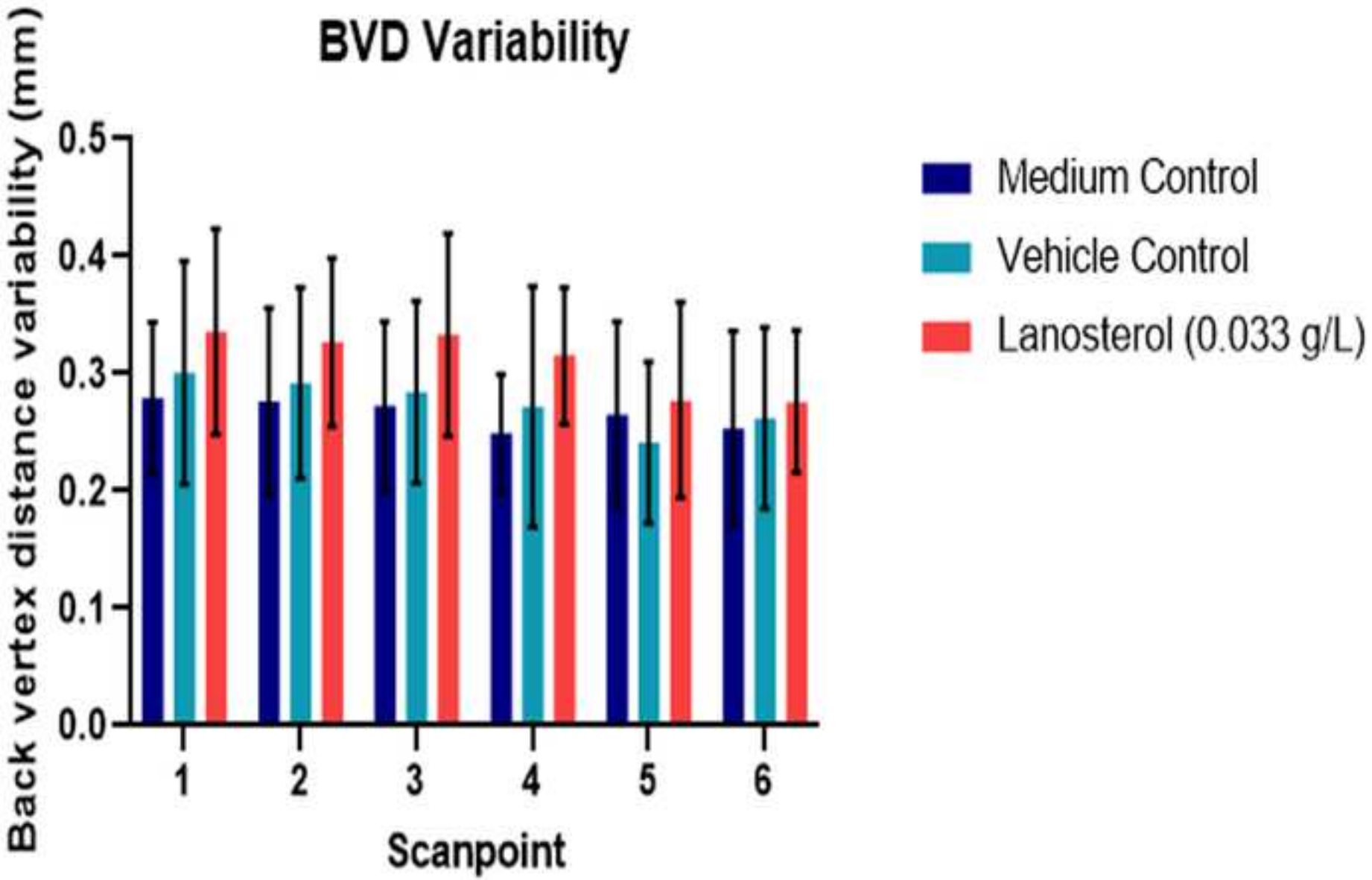

Figure 3

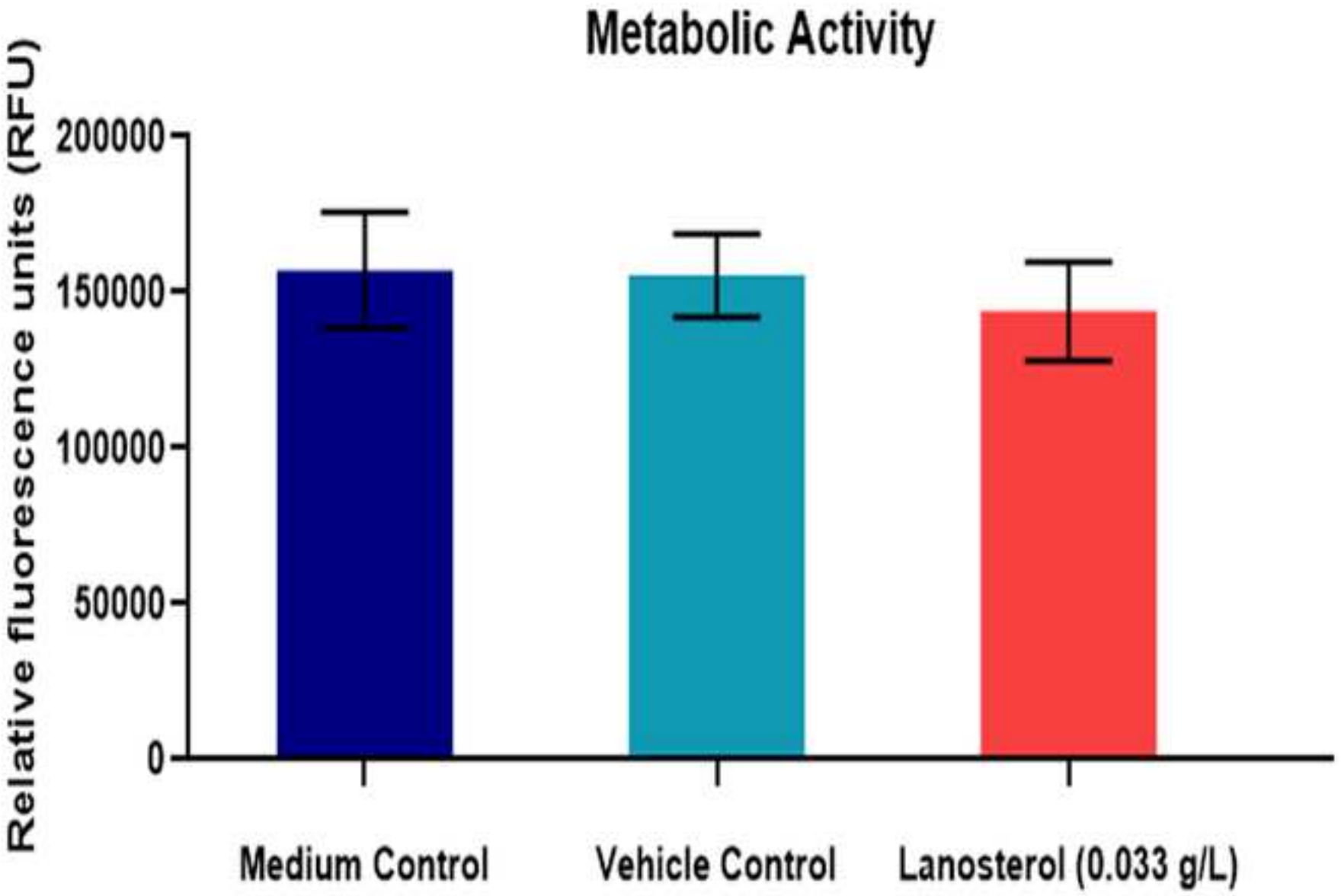

Figure 4

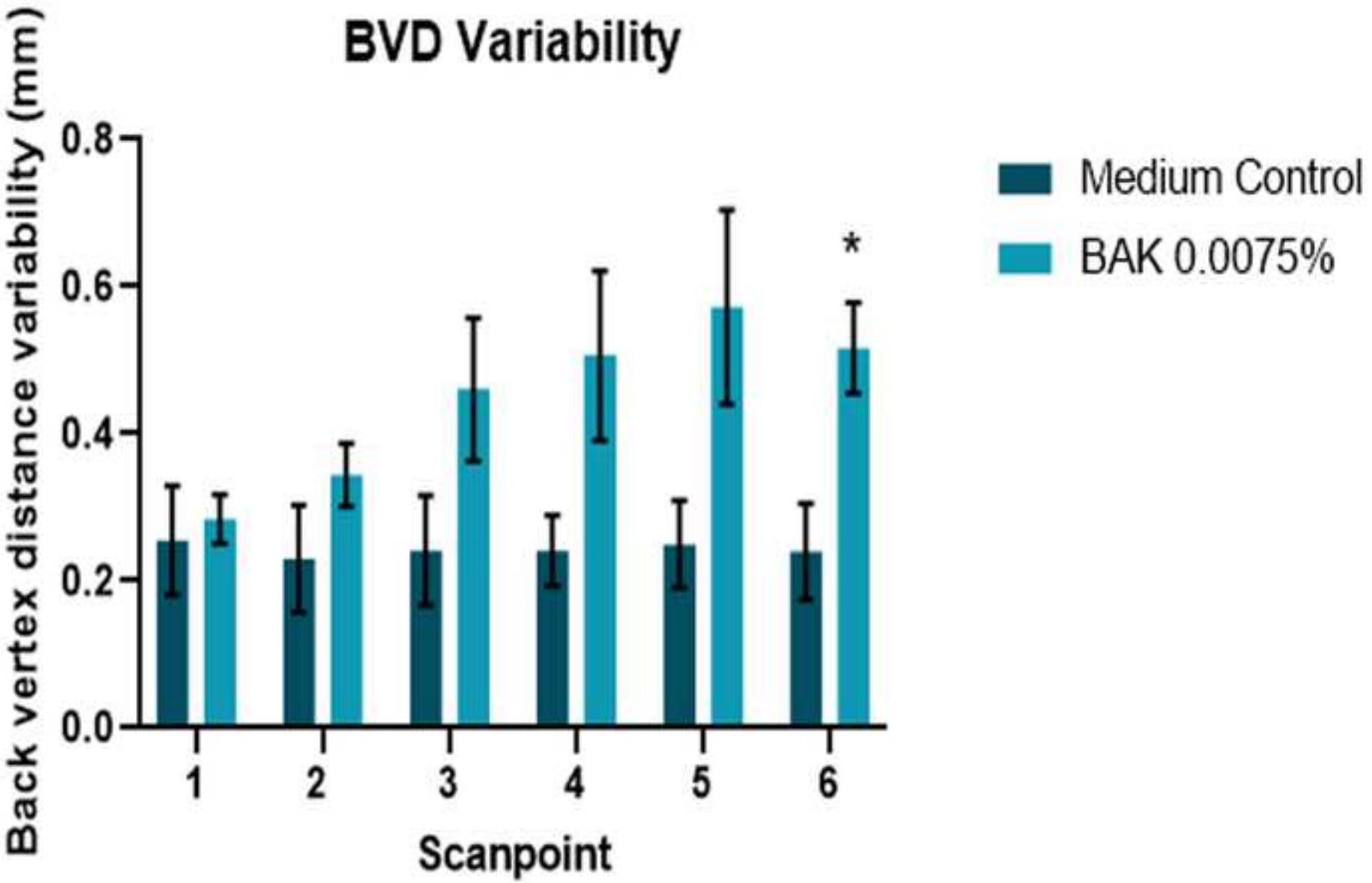

Figure 5

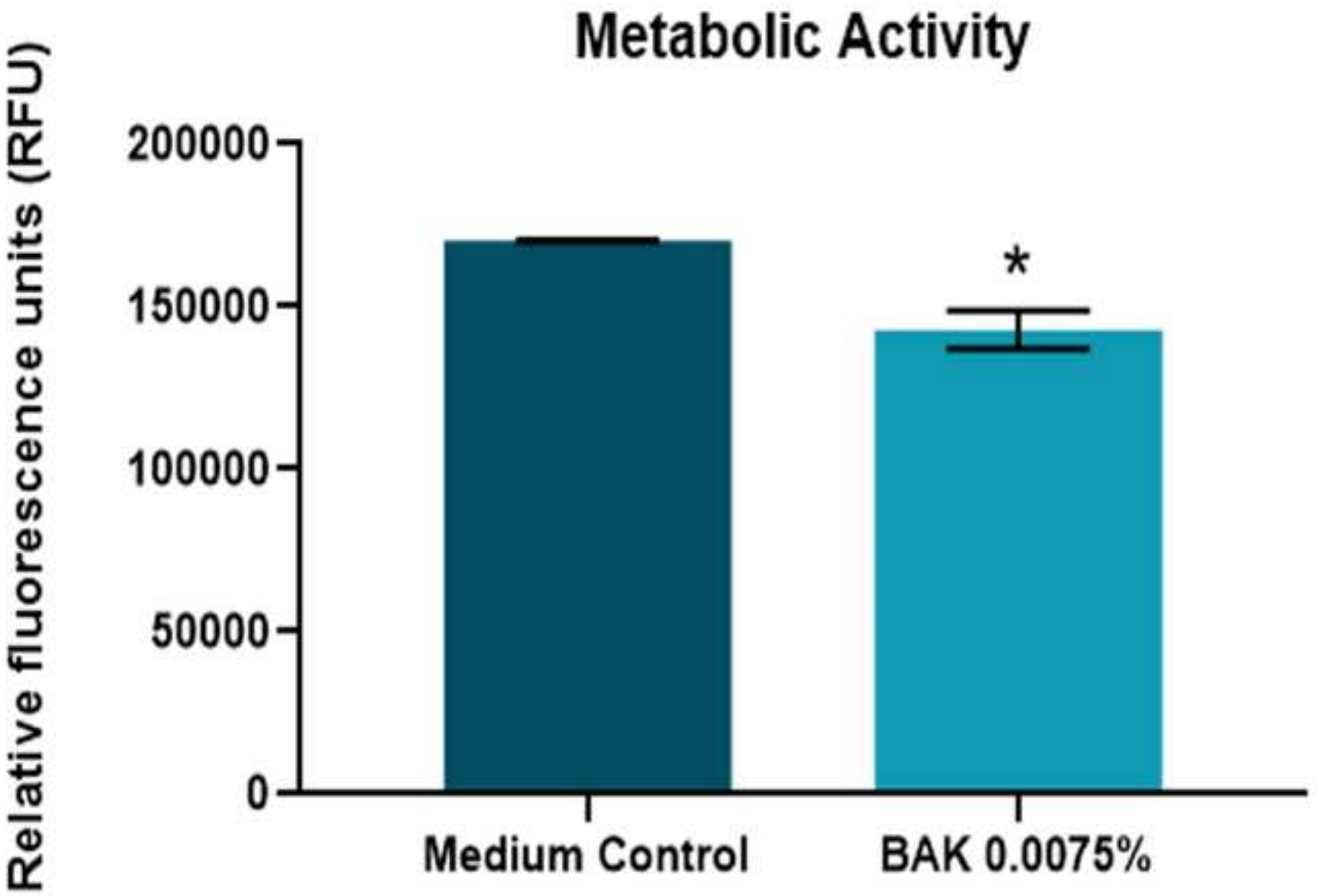

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Table of Materials

Materials and equipment list 18-Nov-2021 (1).xlsx

UNIVERSITY OF WATERLOO
SCHOOL OF OPTOMETRY & VISION SCIENCE
519-888-4567, ext. 33178
optometry.uwaterloo.ca

Nilanjana Saha Ph.D. Review Editor of JoVE
Journal of Visualized Experiments (JoVE)
1 Alewife Center, Suite 200
Cambridge, MA 02140

Revised submission date: November 18, 2021

Dear Dr. Saha:

I am pleased to submit our revision of the manuscript, “Organ culture system for assessing the toxicity of intraocular treatment excipients and pharmaceuticals” by Jordan Rossy, David J. McCanna, Bernard Fresco and Jacob Sivak.

Our responses to the editorial and reviewer comments are contained below for your review.

Sincerely,

Jordan Rossy
Corresponding Author
School of Optometry and Vision Science, University of Waterloo
Phone (519) 888-4567 x32233
Email j2rossy@uwaterloo.ca

Editorial comments: Changes to be made by the Author(s): 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	Some changes made for clarity, spelling and grammar, and submission proof-read and verified through Grammarly.
2. Please provide citations for the following lines: 83-85.	Citations added.
3. Please use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, etc. Use h, min, s, for hour, minute, second. Please use °C for denoting temperature. Maintain a single space between the numeral and (abbreviated) unit.	Submission proof-read for °C and other SI units.
4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Step 1.1: How were the lenses dissected? Please provide all the associated steps. Please mention the animal used, its strain, age, and sex.	Steps describing the dissection process were added. The source of eyes is now described as being bovine, abattoir-provided, non-dairy cows. This is the extent of the information provided by the abattoir. All cows are female, and abattoir-reared cattle are often raised to the same age; the minimum age at which they reach ideal weight for meat production. Yet, this is subject to change based on the needs of the abattoir.
Step 1.3: Section 4.0 describes the optical quality assay. Please correct this.	Corrected to section 4.0.
Step 2.1: How is the compatibility of the chemical compound and solubilizing agent determined?	The use of 2-hydroxypropyl-β-cyclodextrin is reported in the literature to be effective. doi: 10.3390/molecules23051161: "Consequently the more soluble βCD derivatives, such as 2-hydroxypropyl-βCD (HPβCD) and sulfobutylether βCD sodium salt (SBEβCD), are preferred for use in aqueous pharmaceutical solutions, such as parenteral drug formulations, even though both αCD and γCD can be found at low concentrations in parenteral formulations [5]." The current study used this cyclodextrin which enhances solubility in solution.
Step 3.1: Does the chemical compound used in the current study require solution preparation? If yes, please mention how this was done, including the concentration of all the compounds used.	The concentration of the representative test solution for this study has been added. Tests solutions are decided by the primary investigator. Solutions prepared not following the current protocol can still use the exposure method described.

Step 3.3: How was the orientation of the lenses ensured?	This is now described as being performed using a Pasteur pipette and some of the surrounding medium to create a current in the culture chamber which repositions the lens.
Step 4.1: How were the lenses oriented and leveled?	This is now described as using the technique from step 3.3 and visually level.
Step 4.2: How was the culture chamber positioned, and how was the alignment between the pin and the slot made?	This has been reworded for clarity, since the chamber fits only one way into the scanner. This step will be included in the video for additional clarity.
Step 4.3: Please elaborate on the steps used for calibrating the scanner. Please provide all the instrument settings and parameters used to do so.	Additional instructions for beam finders, radial steps, and beam separation specified. Clarification added that the first scan serves to calibrate the scanner with these settings.
Step 4.4: How was the scanning done? How was the desired endpoint determined? How was the scope of the beam set? How many scans were performed for this study? Please provide all the steps required to complete this action item.	The additional instruction to select "scan" to clarify that the scanner carries out this action. The scope is described as being from where the beam begins to the desired endpoint. Selecting the endpoint has been described as, "selecting the maximum distance behind the lens while excluding any apparent interference." The number of steps per scan is now specified in the previous step. Steps 4.4 and 4.6 together indicate that the lenses are scanned twice, along two perpendicular axes – for clarity, the word "second" scan has been used.
Step 4.6 How was the chamber rotated?	Specified as being manual pivoting of the chamber at its base.
Step 5.3: How was the lens rinsing done?	This has been reworded as "perform a rinse by using a pasteur pipette to wash a small volume of unsupplemented culture medium over the lens."
Step 5.5: How was the fluorescence measured?	A fluorescent plate reader automatically measures the fluorescence with the specified settings.
Step 6.1: How was the back vertex distance error calculated? A citation will suffice.	This has been cited and specified as being software-generated.
5. Please provide the composition and concentrations of various compounds and solutions used in the study.	The concentration of the test compound was included in step 3.1. The composition of the culture medium was added to step 1.1.
6. Please remember that our scripts are directly derived from the protocol text. Please include all actions associated with each step. For all the software steps, please make sure to provide all the details such as "click this", "select	Descriptions for software selections were added to steps 4.3 and 4.4.

that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis, including buttons clicked, inputs, screenshots, etc. Please remember that software steps without a graphical user interface (GUI) cannot be filmed.	
7. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol and should also be in line with the Title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.	The steps for the video have been highlighted.
8. Figures 2-5: Please mention the n number of the samples.	The sample size has been mentioned in all of the figure captions.
9. Figure 3: What do CTRL M and CTRL C stand for? What is the concentration of lanosterol used? Please mention in the figure legends.	The terms CTRL M and CTRL C have been replaced in the text and figures. The concentration of lanosterol is now specified in the protocol in step 3.1 and in the figures.
10. Figure 4: What does the y-axis represent? Please mention this in the figure. What do CTRL M, Scnpt1, 2... stand for? Please mention in the figure legend.	The y-axis has been labelled. CTRL M has been removed from the text and figures. Scnpt has been replaced with Scanpoint.
11. Please also include the following in the Discussion in detail with citations: a) Critical steps within the protocol	Additional comments about contamination and dissection quality were added in the discussion of critical steps.
b) Any limitations of the technique	Additional comments about the use of the bovine lens were added to the discussion of limitations.
12. Please add all items (plastic and glassware, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.	Additional items and details added to Table of Materials. Items sorted alphabetically by Excel.
13. Please do not abbreviate journal names in references.	The JoVE reference file was downloaded from the JOVE website and the reference list was populated automatically using this format. If there is a more up to date endnote file for this, kindly provide access so that this can be

	updated.
Reviewers' comments: **Reviewer #1:** 1. What is the period from the death of the animal at the abattoir to the time it is placed in culture? Is this important?	Details were added to step 1.1 – the dissections begin within hours of death and are performed immediately as prevention of contamination and dissection are important.
2. You may want to include the excellent reviews of lens organ cultures: Envir. Tox. Pharmacol 21, 215-221; Experimental Eye Research 170 (2018) 67-75; Journal of Nutritional Therapeutics, 2018, Vol. 7, No. 2; Curr Eye Res. 2018 Oct;43(10):1233-1238. The later 3 articles used rat, porcine and human lens cultures to measure optical properties and select metabolic assays.	Envir. Tox. Pharmacol 21, 215-221; Experimental Eye Research 170 (2018) 67-75; Journal of Nutritional Therapeutics, 2018, Vol. 7, No. 2; Curr Eye Res. 2018 Oct;43(10):1233-1238 These reviews have been cited, excepting the article from the journal of nutritional therapeutics, which could not be accessed.
3. Lines 84 and 85. "…grading lens transparency…subjective." There was a strong correlation between optical density and grade [Experimental Eye Research 170 (2018) 67-75].	The suggested review article also recognizes the subjectivity of photograph grading and the lack of it as an advantage of other methods over photograph grading: "Two major advantages of spectroscopic measurement versus grading of lenses from photographs is that photographic grading only quantifies opacity into 4 to 5 grades whereas spectroscopic measurement provides a continuous grade and is not subjective."
4. Line 347. "The lens laser-scanner assay is unique in that its measurements are directly relevant to the natural function of the lens in vivo." Not unique. Lens absorbance and light scattering is also relevant. See references mentioned above.	This has been revised to "The lens laser-scanner assay measurements are directly relevant to the natural function of the lens *in vivo*." and reference to light scatter was added in the introduction.
5. You may want to give the reader some insights to using other organ-cultured lenses. Advantages and disadvantages could be discussed in a short paragraph. "Compared to human lenses and porcine lenses obtained from an abattoir, rat lenses offer the advantage of being able to control when to they are harvested and the post mortem time to organ culture time is minimal and controllable. Although animal lenses are usually cheaper, more readily obtained and safer to use than human lenses, human lenses differ significantly from animal lenses so the use of human lens models is significantly	Comments about differences between bovine and humans lenses were added as a limitation of the model. Additionally, reference to the use of the laser-scanner with other animal lenses, and factors relevant to the use of animal over human models were added.

advantageous over animal models (Borchman et al., 2004; Truscott, 2005). For instance human lenses diff er from animal lenses in regards to ultraviolet light filters, oxidation with age, protein content, crystalline content, compaction with age, synthesis of ascorbate, antioxidant enzymes (Truscott, 2005) and phospholipid composition and membrane structure (Borchman et al., 2004; Borchman and Yappert, 2010)."	
6. All of the figures require units. For figure 1 the axis's need labels.	Units have been added, as have axis titles for figure 1.
7. Is the standard deviation due to experimental error or the error from lens to lens? What was the number of lenses measured?	The standard deviation seen in the graphs is the error from lens to lens. The number of lenses is now included in the figure captions.
8. Given the standard deviation, what change in parameters is the assay capable of measuring if 5 or 10 lenses are measured?	By increasing the number of lenses from 5 to 10, there would be no change in the parameters of this assay.
Reviewer #2: 1. 1.0 Bovine Lens Culture: The source of the lenses, and how they were removed from the eyes should be stated. The culture medium used, and the pH of the medium, should be stated in the text. Whether the lenses were cultured anterior side up or down should be stated. Some reference to the Table of Materials should be made in the text.	Step 1.1 – the source of the lenses is stated as abattoir-provided, and the order of events for removal from the eye is described. The culture medium and pH are now also stated. The instruction that lenses are ideally cultured in face-down orientation in preparation for the laser-scanner assay. Reference to the Table of Materials is made immediately prior to Step 1.1.
2. Lines 253 and 370: Reference (reference #17) to the use of lanosterol as a pharmaceutical approach to reduce cataract should be deleted. Such use has been disproven in a 2019 publication. See Daszynski DM et al. Failure of Oxysterols Such as Lanosterol to Restore Lens Clarity from Cataracts. Sci Rep 9:8459, 2019.	The language was modified in the original sentence to reflect that this is not proven, using the provided reference. Emphasis is placed on the use of this protocol to evaluate substances which could alter cataract severity.
3. Line 392: I was not able to access reference #2.	The website indicates this is due to a maintenance break.
4. Legends for Figs. 2-5: State the n's.	The n is now stated in the figure captions.
5. Fig. 3: Are there any units for the vertical axis?	There are no SI units for relative fluorescence; these numbers are called relative fluorescence units.
6. Figs. 4 and 5: Define the asterisks. For Fig. 4, was only scan point 6 significant? This should be made clear in the text.	The significant time point for Figure 4 is defined in the text.

7. Line 355: Add "is" before "in".	This has been added ("Since the lens is in…").
8. Line 359: Move "similar" to after "assays".	This has been changed to, "While assays similar to…"
Reviewer #3: Major Concerns: In the introduction I think there needs to be more information on how the cellular properties of the lens actively maintain the transparent and refractive properties of the lens so that stronger links can be made between how compounds by affect lens physiology manifest as changes in overall lens function measured by laser ray scanning	The introduction now includes information from a paper that reviews the properties of the lens which contribute to transparency and its gradient refractive index. Two other studies have also been referenced which support that functional changes, in these cases – scatter, are the product of structural changes.
As stated "Ocular toxicity testing necessarily involves a large battery of tests to determine a broad picture of the safety and tolerance of a compound" - in this regard some information on the through put of the testing system would be interesting to assess the practicalities of using it to do larger scale testing.	Information on the throughput of the system was added following this sentence in the discussion.
Minor Concerns: In my experience handling lenses with metal scoops can compromise transparency	This protocol technique has been used successfully by this group.
Figures 2 &3 should be combined as should Figures 4 &5	The figures are separate to provide more detail and minimize the number of bars per graph.
In Figures 2 &4 the X axis legends are not consistent. I would prefer scan point rather than snpt	The Figure 4 x-axis is now consistent with Figure 2.