**Editorial comments:**  
  
•Grammar:  
-Please copyedit the entire manuscript for numerous grammatical errors. This editing should be performed by a native English speaker and is essential for clarity of the protocol. A small subset of errors is included below (these are only examples).

-Please rephrase the title to remove the personal pronoun “we.”

It has been changed.

-Please correct the grammar in the long abstract. There are comma splices and sentences that need commas for clarity. In addition, please make sure the verb tense is consistent throughout the abstract.  
-2.2 – “recordings” should be “recording.”

It has been changed.

-2.5.2 – “Locate” should be “Place.”

It has been changed.

-2.7 – “Provide” should be “Stimulate”

It has been changed.

-2.7 note – Please use complete sentences. Also, this should be multiple steps rather than a note.  
-3.3 – “duramater” should be “dura mater”

It has been changed.

-3.5 – Please correct “a small piece of gelatin sponge thin layer of gelatin sponge”

It has been changed.

-3.6.1 – “on a breeding cage” should be “in a breeding cage.”

It has been changed.

-4.1.3 – “tip curved” should be “curved tip.”

It has been changed.

-4.2.5 – “Incubated” should be “Incubate.”

It has been changed.

-5.2.1 – Please correct the grammar in the last sentence.

-Discussion – Please correct the following sentence: “In this sense, it results quite interesting to test potential anti-glaucomatous drugs designed to reduce IOP.”

We have changed it and now it appears as follows:

“… In this sense, it is of interest to use this model to test potential anti-glaucomatous drugs designed to reduce IOP…”

•Formatting:  
-There should be a space between steps 5.2 and 5.2.1.

It has been change.

-The ethics statement should be separate from the information regarding housing of animals and type of mice used. It should appear as a note or a step.

The required change has been done and now appears as:

“…6.5 All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research…”

-4.1.5 – The “subbed slides” should appear in the materials table.

It has been included.

-Triton X-100 should always be written out as Triton X-100, and not abbreviated as “Triton.”

It has been changed.

-Please include catalog numbers for the materials in the materials table. Also please include the antibodies in this table.

The table has been completed

•Additional detail is required:  
-3.3 – Is the dental drill used to cut the dura mater? If not, how is it cut?

It has been changed.

-4.1.1 – How are sutures placed?

It has been changed.

-4.1.2 – Substantially more stepwise detail is required. How is the heart accessed for this step? Since this is not to be filmed, a citation would be sufficient.

It has been changed.

-4.1.3 – Please describe how enucleation is performed.

We consider this detail is totally unnecessary

-4.1.4 – Please clarify “Dissect out mice retinas and by means of four radial cuts in the superior, inferior, nasal, and temporal retinal poles”

Done  
-4.1.5 – What same fixative solution? Are coverslips used? Is mounting media used prior to immunolabeling?

Done

The details are in 4.2.6

-4.2.1 – What is frozen for 15 min?

It has been changed.

-4.2.6 – Is a coverslip used?

It has been changed.

-Please define the error in Figure 1 in the figure legend. Is it SD or SEM?

It is SD and it has been added to the figure legend.

•There are instances of unnecessary branding which should be removed:

-Jackson Laboratories should be mentioned in the materials table rather than the description of mice in the protocol.

Done.

-2.7 note – Power Lab

It has been changed.

-6.1 – IPP

It has been changed. DONE

-6.4 – Sigma Plot

It has been changed. DONE

•Discussion: Please discuss the critical steps, potential modifications/troubleshooting, and limitations of the protocol. It is mentioned that there are limitations, but these are not specified.

We have included the following sentence:

“…It is important to notice that the only problem of this approach is the time necessary for the animal to develop the pathology. It is necessary to wait a minimal of 9 months to start to see the changes in IOP and retinal impairment…”

•Length warning: Protocol is at the limit for highlighted material. Any additional detail added may require adjusting the portion highlighted.

We have tried to do our best regarding this but the referees suggested lots of changes that have been included.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

\* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

**Reviewers' comments:**

**Reviewer #1:**

The authors provide many practical details on how to study the course of the eye disease in the DBA2J mouse. It is a good model of experimental glaucoma mimicking the pigmentary glaucoma in humans. The authots have a functional approach with the ERG and a strutural approach with the RGC count. This allows an evaluation of the structure function relation ship.  
Most of the studies evaluating RGC density have been flawed because the investigators did not explore the whole surface of the retina. It has been shown than the degenerescence of RGCs due to ocular hypertension is not homogenous.Therefore it is mandatory to use the system mentioned in this paper using on average about 140 frames to cover the full field of the flat mount retina. Unfortunately many papers did not use this system.Consequently the technique used in this revised mansucript is really the strenght of the study.

All the work of this team has been acknowledeged as a seminal article published in the highest ranked journal in ophthalmology (Progress in retinal and eye research).

We are very thankful to the reviewer's comments. And we greatly appreciate your comments on the manuscript.

**Reviewer #2:**

*Manuscript Summary:*

The authors present a whole variety of methods for glaucoma analysis in the well-known DBA/2J mice. Especially the analysis of the immunostaining is very interesting and useful for readers/viewers of JoVE.

I am personally sorry to say this -as I appreciate the work form this group a lot- but I have severe problems in accepting this manuscript in the state it is now (see major comments).

I regard JoVE as an extremely useful tool to explain methods to other scientist or interested people. Many people including myself get to know and learn new methods using these videos. With this in mind, I reviewed the manuscript.

The methods as they are presented here are given without alternatives, limitations and discussion. And there are limitations (by animal protection law or the be precise they way it is enforced in different countries), which require alternatives and the ERG part requires discussion and alternatives as this is not the best functional test for glaucoma.

*Major Concerns:*

The methods the authors describe here are -without any doubts- the classic scientific gold standard to perform these experiments. I also learned these methods that way.

However nowadays they are in conflict with the animal protection law. Form my personal and the experience from others, section 1, 2, 3 and 4 would not be granted as they are described in the text. (This might differ from country to country)  
To be clear, I used to perform the methods very similar as they are described here but in several animal applications, I wrote over the last years I needed to modify and change them. The authors therefore at least need to address this issue in the manuscript and should present alternatives.

This is a controversial point. The referee is right, things may change among countries, but originally this contribution was suggested by JOVE being the journal particularly interested in the way we proceed methodologically speaking, which is highly reproducible. If we modify this methodological aspect we would move from the original perspective and moreover we would come into other protocols we do not perform.

The issues in detail are:

- Ketamin / Xylazin anesthesia is generally regarded as outdated. Alternatives should be given.

We are very sorry, but we disagree with the reviewer on the use of ketamine/xylazine. Ketamine / Xylazine anesthesia cocktail is widely used in rodent research or in combination with others drugs (**Gargiulo et al., 2012**). When it is browsed through scientific databases it can be found that its use has been growing since 2000 till 2006, together with isofluorane , in all the research areas with rodent (**Stokes EL, Flecknell PA, Richardson CA. Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. Lab Anim. 2009 Apr;43(2):149-54.**). We think ketamine/Xylazine anesthetic mix cannot be regarded as outdated. It just one more option to use, like ketamine/xylazine/ acepromazine, ketamine/xylazine/ buprenorphine, ketamine/dexmedetomidine, ketamine/medetomidine, isofluorane, or others. Induction of general anesthesia in mice can be achieved by a variety of drugs and techniques (**Flecknell, 1989**). Each researcher has to choose the best option to his experimental procedure according to the local and international regulations that applied in his institution.

The most commonly used anesthetics in mice include the injectable agents avertin, pentobarbital, and ketamine, which are often combined with other agents such as acepromazine, xylazine, diazepam, several narcotic analgesics, and the inhalation agents halothane, isoflurane, and sevoflurane. Compared with injectable techniques, inhalation anesthesia provides greater safety, particularly for prolonged procedures, due to a lesser cardiovascular depression, a reduced impact on liver and kidney functions, and because it promotes rapid recovery and allows quick adjustments and easy maintenance of a steady anesthetic depth. However, inhalant agents foster respiratory depression (particularly in the presence of respiratory diseases), myocardial depression, vasodilation, and hypotension (Paddleford, 2000), exhibiting weak analgesic effects.

Finally, compared with injectable drugs, the modern inhalation anesthetics require complex and expensive equipment such as precision vaporizers and flowmeters, specific breathing systems, and efficient scavenging systems to prevent pollution (**Gargiulo S, Greco A, Gramanzini M, Esposito S, Affuso A, Brunetti A, Vesce G. Mice anesthesia, analgesia, and care, Part I: anesthetic considerations in preclinical research. ILAR J. 2012;53(1):E55-69).**

We will give a note with references for anesthesia alternatives (**Gargiulo S, Greco A, Gramanzini M, Esposito S, Affuso A, Brunetti A, Vesce G. Mice anesthesia, analgesia, and care, Part I: anesthetic considerations in preclinical research. ILAR J. 2012;53(1):E55-69).**

- However, in special cases (ERG measurements) there is a chance to get allowance for that. The authors should provide the user with useful information to get the allowance.

To perform an ERG is mandatory to induce an anesthesia degree to provide good and adequate immobilization to carry out the ERG measurements without any kind of interference between the stimulus and the eye. And ketamine /xylazine mix provide it. Inhalation anesthesia is administered by anesthesia machines and delivered by breathing system that can interfere with the ERG measurements. The reference electrode localization in the mouth could be a serious inconvenience to the inhalation anesthesia administration too.

- There is no need to anesthetize mice for IOP measurement. A sedation is sufficient.

We agree with the reviewer. However, IOP is measured under general anesthesia in most studies using mouse models (**Ding et al., 2011**), maybe because accurately and reliably measuring IOP is critical in glaucoma investigation. Others alternatives to general anesthesia to measure the IOP are sedation or anesthesia at low dose, immobilized conscious mice by restrainer and, even, mice with behavioral training to perform awake IOP measurement without immobilization (**Ding et al., 2011**). But in the case of DBA/2J the development of intraocular hypertension is associated with the age of the animal, and these older animals (more than 12 months old) could be more susceptible to stress by manipulation and usually the time course of a study with DBA/2J mice is spread almost a year.

- Neither craniotomy nor the removal of the brain cortex is needed to label retrograde. No allowances are therefore given any more to perform retrograde labelling with craniotomy and removal of the brain cortex in this country. Please also give alternatives (injections).

As suggested by the reviewer, there is other alternative method to RGC retrograde tracing from both superior colliculi and it can be achieved by multiple stereotactic injections (Barnstable and Drager, 1984; Siddiqui et al., 2014; Soto et al., 2008) but, if it is not done properly may lead to regions of the retina left untraced. Other alternatives methods are from the intact optic nerve, that is without severing the optic nerve (Nadal-Nicolas et al., 2014, 2015), or by a single stereotactic injection in the optic tract (Nadal-Nicolas et al., 2015).

We are very pleasant to give all these alternatives to the reader. But we have to keep in mind that we are going to study the functional integrity of RGC retrograde transport. And we need to place the tracer on the retino-topic projection nuclei available to the RGC axon terminals. One characteristic of DBA mice, as others mouse glaucoma models, is that when the animal expresses the pathology there are retina areas where the retrograde transport is compromised, showed by untraced retina areas or regions, and you need to be secure to have a good, complete and homogeneous tracer available to the axonal terminal and that the areas devoid of tracer are not artifact.

- There is no need to transcardially perfuse a living mouse to enucleate and fixate an eye and perform a whole-mount. Mice can be killed and then the eyes can be fixed and whole-mounts can be prepared or the other way around. There is multiple publications performing whole-mount preparation these ways. Please mention these alternatives and discuss them.

We are very sorry but we are disagreeing with the reviewer comments on this point. The animals are transcardially perfuse after a deep anesthesia with an overdose of 20% sodium pentobarbital and when all the nociceptive reflexes are abolished the perfusion is done. On the other hand, the main reason to carry out a transcardial perfusion is to remove the blood cells from the retina vascular system to avoid as much as possible the presence of autofluorescence elements or background that can interfere the RGC quantification by automated methods.

- I also do not regard STR as a hallmark for glaucoma. Yes, there are differences. However, the authors also should address, this in the introduction and discussion, that there are better (although harder to perform) hallmarks: VEP!

The authors mix the terms RGC death and retinal cell death often in the text. Especially when it comes down to the ERG part. For the unexperienced reader this might be misleading as there is definite difference. Please straighten this out. This is especially crucial in Line 438. An ERG does definitely not "fully confirm the lack of functionality"

We agree and we have changed retinal by ganglion. We have also removed the word fully as indicated in the comment.

The authors should also mention that there are other control strain than the C57/BL6J mouse. Especially as the C57/BL6J is not regarded as the best strain for comparisons.

We agree with the reviewer with respect to the existence of different controls. DBA/2J-Gpnmb+/SjJ provides a genetically matched control for DBA/2J mice, this coisogenic strain has a functional allele of Gpnmb which does not develop glaucoma. In spite of this comments, Porciatti et al, 2010 reported a comparison between different strains including C57BL/6J, DBA/2J and DBA/2J-Gpnmb+/SjJ and their results showed different characteristics in these mouse strains. Furthermore, retinal ganglion cell population is reported to be larger in DBA/2J than in C57BL/6J mice,(Williams RW , genetic and enviromental…)

16 out of 31 citations are self-citations. There is so much literature about the model and these methods available. Please cite and mention also the work of others in this field.

There are no so many researchers working on ERG and this animal model. Indeed the number of papers included in the text by the three different groups participating in this study are additive and therefore it is not strange that 50% of the reference belong to any of the groups. What is important is that all the quoted references (ours or not) are necessary for a better understanding of the manuscript, particularly when this type of paper is read by not specialist in the topic.

*Minor Concerns:*

Instead of "mouse" I would use DBA/2J mouse a key word.

It has been changed.

Introduction:

Regrettably, there are almost no citations! See also comment above. We all depend on being citied, so please respect and appreciate the work of the people you mention and cite them.  
Personally, I was really wondering who uses the application of phenol to induce IOP. I never heard of this and could also not find this in the literature.

We have included several references regarding this.

The first paper describing the DBA/2J mouse as a glaucoma model is from 1998. This is not "recently" (lines 152 and 156).

Done.

Methods:

The order of methods should be changed according to the results and figures. ERG is always last there.

The referee is right and we have changed the figures according to the text.

Line 203. No surgery was performed afterwards.

Line 209/210. Should be transferred to Line 217

Line 265-267: A link where to find these guidelines would be nice.

We have included a new reference

Line 312: define "normal"

It has been changed.

Line344: should be "fresh PBS" and "for 10 minutes"

Done.

Line384: This is a method article: The procedures should be in the article somewhere (or supplements)

The method has been already described elsewhere and moreover the lack of space obliged us to put the mentioned reference.

Line391: Please give more details about the macros

The macro designed to count RGC has been previously published (Salinas-Navarro et al., 2009). In brief, we used macro language to apply a sequence of filters and transformations to each image in order to clarify cell limits and separate individual cells for automatic cell counting. In a first step, the images are converted to 8-bit gray scale images. Illumination aberrations caused by the microscope optics are removed by the software flatten enhancement filter which evens out the background variations. This was followed by enhancement of the edges of the cells using the large spectral filter edge+ command, which extracts positive edges (in this case fluorescent stained bright cells) from the dark background. A setting of 8% (kernel size 20×20) was sufficient to enhance the cell edges making detection simpler. Large spectral filters are used where large kernels are required and cut down on the processing overheads. Small artifacts and noise are removed by running three passes of the median enhancement filter (kernel size 3×3). Cell clusters are then separated by two passes of the watershed split morphological filter which erodes objects until they split and then dilates them until they do not touch. The cells in each image are counted using predetermined parameters to exclude objects that are larger than 300 μm2 or smaller than 7 μm2. These parameters correspond to the largest or smallest individual OHSt-labeled object detected as RGC. Finally, each count was exported by dynamic data exchange to a spreadsheet (~~Microsoft~~~~®~~~~Office Excel 2003, Microsoft Corporation, Redmond, WA, USA).~~

Line395: see Line 384

??

Line 426: Should be: Retinal Ganglion Cell Death

It has been changed.

Line 427: It is a bit cynical to call "blindness another feature of glaucoma pathology".

We have changed it to “…consequence…”

Figure Legend:

Line 449/450: I also see measurement points of 6 and 12 months. Please clarify.

We commented the most representative changes when they occured nevertheless we performed a time course along 15 months.

Line454: According to the following text the mice are aged 6, 12 and 15 months and not only 15 months.

See previous comment.

Figure 3: Would be helpful if the A and B would also have a legend in the Figure, which color belongs to which line. C and D also here a bit text in the figure would help: 6 month above C 12 months above D. Explain circles and triangles.

The explanation has been done in the figure legend.

What are the last two pages "comments / Description" meant for?

We do not understand what the referee is talking about.

Please proofread this article once more. There are still major unmentioned language flaws.

Done

*Additional Comments to Authors:*  
N/A

**Reviewer #3:**   
*Manuscript Summary:*   
N/A  
  
*Major Concerns:*  
RGC counting based on OHSt retrograde tracing is fundamentally flawed, as it assumes that axon transport is intact in glaucoma models. Instead, axon transport is well known to be impaired early in the disease, including DBA/2J glaucoma. Thus, loss of OHSt-RGC staining cannot distinguish between RGC loss and loss of axon transport.

We think that there is a misunderstanding.

Of course, it has been showed important alterations in the retrograde and orthograde axonal transport in RGCs associated with OHT (for a review, see Vidal-Sanz et al., 2012).

In the DBA/2J mouse the fact that RGCs are identified with OHSt applied to their target regions is that this technique does not reflect RGC survival but rather an alteration of the retrograde axonal transport. Indeed, Brn3a immunodetection is used to determine RGC survival.

The presence of the tracer within the RGC bodies implies an active retrograde transport from the axon terminals in the SCi all the way back to the retina. It is possible that the absence of retrograde-labeled RGCs observed in DBA/2J mice retinas is related to a functional impairment of the axoplasmic flow, as has been observed following other types of experimental glaucoma models (Vidal-Sanz et al., 2012).

With this experimental approach it is possible to identify RGCs maintaining a functional retrograde axoplasmic transport, while Brn3a immunohistofluorescence is used to identify surviving RGCs. This observation further supports the concept that not all surviving RGCs retain normal physiological properties.

The pSTR does not seem an adequate method to assess RGC dysfunction. Several studies show that another functional measure ─the PERG─ is extinguished before 12 months of age in DBA/2J mice. Figure 3 of this study instead shows only a moderate reduction of pSTR between 12 and 15 months. According to current literature, at this age a large population of RGCs is already lost. So, what is the purpose of recording the pSTR? RGC function would be expected to deteriorate before RGC loss. This should be discussed

The referee is right about the importance of this approach (PERG). We are now fixing the conditions to perform PERG although by now pSTR is well established in our group and others to determine retinal ganglion cell degeneration.

*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A