We thank the editor and all the reviewers for their comments. We have revised the manuscript based on all comments (see accompanying manuscript file where all the changes have been tracked) and provide a detailed answer to comments and concerns raised by editor and reviewers below.

**Editorial comments:**  
  
*1. More detail is needed in a number of steps:*

We have now given more details in a number of steps related to the retina dissection and stimulation taking into account the very helpful comments by reviewers 2 and 3.  
  
*a) 2.3: How are mice euthanized? How do you dissect the eyes with tweezers?*

This information is now provided.  
*b) 3.1: How is the software configured? (ie, what are you clicking to configure the software in this example?)*

We have now given clearly the parameter values related to data acquisition as well as stimulation parameters in units typically used in a commercial *in vivo* system. However, we feel that giving details of how to configure a specific software will not be helpful as different *in vivo* systems have different software interfaces.

*c) 3.2: Please give an example of stimulus parameters to use.*

This information is now provided.  
  
*d) 3.3: How long should the experiment last? Are you recording the signals? (“Follow” is vague.)*

We have changed “follow” to “monitor” and give a rough time scale for typical experiments.

*e) Line 204: Typo—should be Figure 2 not Figure 7*

Thank you, this is now corrected.  
  
*2) Figure 2 legend says the red lines in 2D are the samples in APB w/o barium, but the labels in the figure say the opposite. Either the legend or the figure labeling is incorrect.*

We apologize for the mix up in the color scheme in Figure 2D, the colors are now corrected in the Figure.  
  
*3) Formatting:   
a) In vivo and ex vivo should be in italics throughout.*

We changed the formatting as requested.  
  
*b) First 2 lines of protocol (above the ethics statement) should be a note in section 2.*

The information about the housing and dark adaptation of the animals is now part of section 2.3. The mouse strain information is now in the Representative Results section.

*c)1.4 & 1.5 plus part of 1.3 should be substeps 1.3.1, 1.3.2 & 1.3.3 under 1.3) Prepare and test the specimen holder.*

We have revised the numbering according to the reviewer’s suggestion.  
  
*d) 2.4: Split into two steps*

We have now split 2.4 into two steps.  
  
*4) Discussion: Which protocol steps are most critical?*

We describe now the most critical steps in the first paragraph of Discussion.  
  
*5) References: Some DOI missing. Please make sure that your references comply with JoVE instructions for authors. In-text formatting: corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text of the manuscript. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source.****Volume****(Issue), FirstPage – LastPage, doi:DOI, (YEAR).]*

We have used a recent JoVE endnote style provided in JoVE website and the references have been imported directly from PubMed (NLM) database. Some of the older references might not have DOI or at least we couldn’t find them.

*6) 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.  
  
7) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.   
  
8) 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.*  
  
  
**Reviewers' comments:**  
  
**Reviewer #1:**

We thank reviewer #1 for the supportive comments and have addressed all minor concerns (see below).

*Manuscript Summary:*   
*This is a timely and important technique that addresses the major limitation of conventional in vivo ERG, its reproducibility.  
This approach not only improves the stability of the retinal field potential, but also enables the testing of different pharmacological agents.   
  
Major Concerns:*  
*none*  
  
*Minor Concerns:*  
*I am not sure if the authors mention one of the additional components of the ERG, the oscillatory potentials (OPs). These are the oscillating deflections riding of top of the b-wave and reflect the activity of the neurons in the inner retina, presumably inhibitory amacrine cells. Does this technique also reveal this important component of the ERG? From provided illustrations, it's hard to discern their presence. Please, comment.*

This is a very good comment and we have been puzzled why OPs are not so prominent under *ex vivo* conditions. In contrast, the b-wave driven by ON bipolar cells appears very strong and stable indicating that inner retina signaling can be preserved *ex vivo*. Though beyond the scope of this paper, developing experimental protocols that preserve OPs and enable their electrophysiological characterization *ex vivo* will be a very useful addition to the range of studies possible with this technique. We have now discussed the lack of OPs in our *ex vivo* recordings in the Discussion.  
  
*Additional Comments to Authors:*  
Great work and highly appealing to many researchers studying retinal function.  
  
  
**Reviewer #2:**

We thank reviewer #2 for the helpful comments and have used them to improve the quality of the manuscript by giving more details about the sample preparation and the design of the specimen holder.

*Manuscript Summary:*   
*In this article authors describe the use of isolated retinal tissues to record light responses from rod- or cone- or mixed photoreceptor driven retinal circuits. The experimental details regarding the isolation of the retina and mounting of tissues into the chamber are rather scant.*  
*Major Concerns:*  
*Overall it appears as though a great simplification has been given. Is the isolated retina chamber to be purchased? The website given shows $11,000 for this part. No dimension or schematics are given, making this appear as a commercial for the product rather than a technique that can easily be repeated by readers of this article. Authors should include the important and essential characteristics that would allow readers to make their own simple chambers.*

See our reply below.  
  
*Page 3. Lines 103-106. The description of the specimen holder is extremely sparse and without a schematic and some dimensions, cannot be created given the level of the description. The size and curvature of the domes, the materials and methods for inserting the electrodes all need to be clear. The mechanism by which the retinas are held in place and the electrical isolation of the two sides must also be made clear.*

We have now referred to the mechanical drawings published recently by us (Vinberg et al. 2014, Vision Research). In addition, the specimen holder and electrodes are commercially available. We have provided additional details about the sample preparation and retina mounting procedures. The specimen holder has been designed to keep retinas in place without any extra support and following our description here with the available specimen holder it is very unlikely that the retina will displace during the experiment. We have also explained why and where the O-rings are in the specimen holder. It is an important feature of the holder that allows recording of low-noise and large amplitude *ex vivo* ERG signal.  
  
*Minor Concerns:*  
*Page 4 lines 138-9. Also unclear and poorly written,*

We have revised this section in an effort to clarify it.  
  
*Page 4 line 143, sentences as written suggests we immerse tweezers in the media. Are the dissections taking place within the large petri dish in order that the retinas be mounted on domes? If so state that the retina must remain always within the fluid of the perfusion media during dissection and mounting.*

We have clarified this part of the manuscript.  
  
*Should the media be oxygenated prior to pouring it? (line 138)*

We have not oxygenated the media here for the dissection. We make this clear now in the manuscript.   
  
*Explain the general stages of preparation…. Why is the petri dish necessary, how long will the chamber be in there?*

These steps have now been clarified.  
  
*A schematic of the upper and lower retinal holding chamber is necessary. Some sense of scale as well.*

See above.  
  
*Line 153: Which side of the retina touches the dome? How can you tell which side is which? Is the RPE remaining on the retina? If the RPE supplies the retina with chromophore and lights are used to perform the mounting, how will the visual pigment be regenerated.*

The retina mounting procedure has been revised to include more details. RPE will mostly detach from the retina during the dissection process (at least for mouse) and thus the RPE-mediated visual cycle is not functional during the *ex vivo* recordings. We do not find differences between using dim red light or IR light for preparation suggesting that the dim red light used during the mounting etc. does not bleach significant amount of the pigment. For recordings from dark-adapted retina, the intensities of test flashes used in a typical experiment bleach only a negligible fraction of the pigment so that RPE-driven regeneration is not an issue. In addition, as we have shown previously, cone pigment regeneration can still take place in the isolated retina via the Muller cell visual cycle. This is now mentioned in section 3.2.  
  
*There appears to be black o-rings, what size are these and what, and how do they accomplish their purpose?*

See above.  
  
*Page 5 Line 198 The Ames vs. Lockes buffer results are in contrast to published works on single rod photoreceptors. Please reconcile the difference of these two approaches. Either one is less sensitive, or the retinas/cells have been damaged. The recovery times are substantially faster in Ames.*

This is already discussed in Azevedo et al. paper (J Neuroscience 2011 Mar 9;31(10):3670-82) where both single cell and transretinal recordings were used. We have mentioned this work in the Discussion but feel that further discussion would be beyond the scope of this manuscript.   
  
*Fig 3. The typical isolated cone driven ERG is about 35% of the dark adapted ERG (b-wve), yet in your Fig 3 b, it is only 10%. Can you explain this very small cone response?*

This is now explained in Discussion.  
  
*If you block potassium flux from Muller cells with barium, then isn't there an elevated potassium level in the retina that will alter the light response?*

All pharmacology has potentially some side-effects. There are always pros and cons related to any technique. However, several publications indicate that transretinal ERG with barium provides comparable results to single cell recordings. One possible explanation is that the excess potassium is being removed from the isolated retina by the perfusion solution, i.e. the extracellular potassium concentration is set by the perfusion media.  
  
  
**Reviewer #3:**

We thank reviewer #3 for the extensive specific comments. We have revised the manuscript accordingly and provide explanation for the more significant changes below in reply to the reviewer’s specific comments. We now provide substantially more details about the light stimulation that should help to choose correct stimulation in *ex vivo* recordings performed with different commercial in vivo ERG systems. We decided to remove the conclusions about fractional sensitivity and kinetics due to reasons explained below.

*In this manuscript the investigators describe how commercially available and widely used in vivo electroretinography setups could be with moderate additions/changes used for simultaneous ex vivo transretinal recording from two isolated retinas. Ex vivo ERG allows pharmacological isolation of components originating from certain cell types as well as testing of the efficacy and safety of potential therapeutic drugs. There is rapidly growing interest towards both of these, and therefore the description of the ex vivo ERG protocol may be of great interest and useful for a wide audience and especially to those already having an in vivo ERG setup. The protocols and procedures are explained clearly in the text (except light stimulation, see below), and it is my understanding that the reader should be able to repeat the experiments in her/his own setup based on these descriptions.*  
Specific comments:  
*Lines 113-4. It might be worth emphasizing here that even small bubbles in the electrode space can increase noise and therefore should be avoided (though this comes less explicitly on lines 180-1).*

This issue is now mentioned in section 1.3.3 and in the Note at the end of section 3.

*Lines 124-8. It might be useful to say that in order to keep the perfusion rate relatively constant the perfusate bottle should be placed high enough above the specimen chamber level, e.g. ten times higher compared to the change of solution level in the bottle during the experiment (Fig. 1D may give a wrong impression on the appropriate location of the bottle).*

This is a very good point that we also considered during the development of the adapter. We now discuss explicitly how significant gravitational drive is achieved with specific *in vivo* systems in section 1.5.

*Line 148. I find the sentence "Cut a small slit between the retina and sclera" confusing. What is meant with that?*

In an effort to clarify, this sentence was replaced by: Cut a small incision from the top of the eye cup towards the optic nerve by keeping the scissors between the retina and sclera in order to keep the retina as intact as possible.

*Lines 146-151. Retinal pigment epithelium is not mentioned at all. How is the retina isolated from the pigment epithelium?*

Yes, the retina detaches from the pigment epithelium during dissection. This is now mentioned in section 2.5. Dissection details. As now discussed in section 3.2, this does not affect the light responses in a typical experiment as the amount of pigment bleached by the test flashes is negligible.

*Lines 141-166. No attention is paid on the time spent in the isolation procedure. Is it critical that isolation is completed very rapidly? How does it affect the state of the retinal cells if the isolation is done "slowly"?*

This is an important point that has been now addressed in section 2.5.

*Line 170. The acquisition frequency band is not given right. I assume that it was intended to tell that the acquisition frequency range is from 0 to 1-10 kHz (taking into account that on line 178 the reader is given advice to follow the baseline drift).*

The confusing phrasing in the original manuscript has now been corrected. We meant that any sampling frequency between 1 and 10 kHz can be chosen.

*Lines 169-182. Practically nothing is said about light stimulation. In ERG all the photoreceptor cells in the active recording area should be stimulated equally. The authors should give some general description how this can be achieved in the commercial in vivo setups and how the level of photoreceptor stimulation should be estimated. How do the possible reflections in the recording chamber affect the homogeneity of stimulus light at the retinas and how can the reader achieve appropriate light stimuli at each of the two retinas?*

The original idea was to give stimulus in units that are independent of stimulation light (and we should have mentioned that we used green 530 nm light) but we agree that in this type of article readers should be given stimulation parameters that can be directly used in their *in vivo* system’s software. We have now clarified how to choose stimulation in comparison to *in vivo* recordings and give approximate range of values in photopic luminous energy (Cd s/m^2). We also refer to our recent paper which includes extensive explanation of how photopic luminous energy can be converted to pigment isomerizations which is ultimately the brightness that photoreceptors detect. Recording from two retinas simultaneously is of course only possible with *in vivo* systems that can collect data simultaneously from both eyes of the animal (e.g. LKC and Ocuscience). The location of the retinas in the *ex vivo* experiments is very close to the location of the eyes of a live animal in the Ganzfeld sphere during *in vivo* recordings. Therefore, the incident light uniformity and energy are comparable between the two methods. The reflections in the specimen holder could indeed affect the pigment isomerizations as discussed in Vinberg et al., 2014. To address this issue, the commercial adapter includes a black cover with apertures above the retinas which reduce light scattering and facilitate accurate calibration of light strength.

*Line 206-8. The sentence "However, fractional sensitivity, kinetics and stability were comparable across all perfusion conditions used here" is confusing. It sounds to me that this sentence refers to the data of Fig. 2D-E, i.e. in the presence of both APB and barium, not to "all perfusion conditions". Further, the terms "fractional sensitivity, kinetics and stability" lack attributes they are referring to. The authors should formulate the sentence more precisely to make it understandable also to readers not familiar with photoreceptor physiology.*

We agree that we were not clear enough with the definition of fractional sensitivity and kinetics. In addition we didn’t perform a comprehensive set of experiments that would allow us to draw conclusions about sensitivity that somewhat varies between the experiments. We think that there actually might be about 2-fold higher fractional sensitivity in Ames as compared to Locke’s and Ringer (although kinetics as judged from time-to-peak of a dim flash response appears invariant across all perfusion solutions). However, we feel that the subtle differences in response properties under different media are outside the scope of this paper. To simplify the text, we have removed the related conclusions about fractional sensitivity and kinetics.

*Lines 226-236. The values given for flash strengths are not useful without telling the stimulus light composition. Please, give that for both the data in Fig. 2 and Fig. 3.*

See above.

*Lines 227-231. The colours in the text do not correspond to those in Fig 2D. Switch the colours of the curves in the figure (black and red).*

This is now corrected.

*Lines 233-237. It is told in the text that the recordings shown in Fig. 3 are cone responses. To me it seems that the recordings show timely overlapping components from cones and bipolar cells, i.e. APB was not present in the perfusate. I would like to suggest the authors to add double flash cone data from recordings with APB present in order to show also "pure" cone responses.*

We indeed isolated here cone-mediated responses in the absence of APB because there is already a comprehensive study by Heikkinen et al. 2008 about how to isolate pure cone responses in mouse transretinal recordings. We cite that work but stick with the original response family containing the b-waves. We have made the text more clear about what were recorded.

*Line 241. The first sentence in Discussion gives me the impression that the two retinas recorded from were perfused with different solutions. I cannot say whether it was the case in reality, but it might be worth emphasizing this possibility in the text.*

In this study we did not perfuse two retinas from the same mouse with different solutions. This is now explicitly stated in the Discussion but as suggested by the reviewer we now mention this possibility.

*Line 265. See the comment concerning lines 206-8.*The discussion about fractional sensitivity is now removed (see above).

*Minor:  
On line 7 in the Abstract, I find the words "different standard" needless.  
On the last line of the Abstract, I would replace "different" with "certain".  
Line 40. I find the words "different standard" needless.  
Line 248. I would replace "through" with "above".  
Line 263. I would replace "meant" with "intended".  
here is that any effect of channel modulation in mouse rods by calcium is quite small*

We have done these revisions.  
  
  
**Reviewer #4:**

We thank reviewer 4 for the comments and have revised the manuscript as suggested. It is true that the original manuscript did not clearly state whether electrodes are included in the commercial system and that was because all the commercial references were removed in the editorial process from the originally-submitted text.

*Manuscript Summary:*   
*The authors describe a nice and easy method for recording light responses with high signal-to-noise ratio from isolated retinas using commercially available equipment and software. The usefulness of such "ex vivo" ERG recordings from intact retinas has increased enormously since they were first introduced in the late 1960's thanks to the analytical understanding of the components, enabled by insights from more invasive (and more demanding) techniques. It now provides an attractive possibility, e.g. for fast testing of transgenic mouse strains, or for successful study of human retinas, with respect to the functioning of rods and cones as well as other retinal elements. Parallel recording from two retinas as introduced by the authors minimizes biological variation in treatment/control comparisons and reduces the number of experimental animals used.*  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
*I have a few minor comments:  
1. Abstract last sentence: It is not the method here described that is used to remove specific ERG components, but pharmacological treatments.*

The last line of the Abstract was revised to address this concern.

*2. Introduction: I think it would be worthwhile to mention how the ERG signal arises as a field potential mainly reflecting light-induced changes in radial extracellular current components in the resistive retinal tissue.*

This is now mentioned in the Introduction.

*3. Lines 52-53: "the ERG signal" should be "the ERG response to the onset of light"  
4. Line 65: "modalities" is not a good expression: maybe rather "recording configurations".  
5. Line 66: "Transretinal ERG from the intact, isolated retina (ex vivo ERG)…"  
6. Line 67: "Few studies" should be "A few studies"*

All of these (3-6) changes have been now made.

*7. Lines 80-81: although the "power" of the b-wave is provided by Müller cells, it sounds somewhat misleading to refer to this (and the slow PIII) as questions of Müller cell function.*

The text describing the use of *ex vivo* ERG to assess Muller cell function has been clarified. We have now described the involvement of Muller cells in generation of the negative slow PIII component that is prominent in the *ex vivo* ERG signal (see Figure 2D). In this way it is explicit what we mean by suggesting that *ex vivo* ERG can be used to study Muller cell function in the intact retina.

*8. 8. Line 246: "custom-build" should be "custom-built".  
9. Line 263: "the" is missing in "meant to isolate the photoreceptor component…"  
10. Line 288: "barium" should not be capitalized  
Throughout: Since the names of the originators of the solutions used are Ames, Locke and Ringer, the proper spelling of the respective solutions is Ames' solution, Locke's solution and Ringer's solution (or plainly Ringer).*

All of these changes (8-10) have been now made.

*The procedures appear to be described in sufficient detail, although this is difficult to judge by just reading. E.g., presumably the electrodes used in the ex vivo adapter are included in the commercial system and need not be separately mentioned?*

The electrodes are indeed included in the system and significant amount of effort has been used to design electrodes that are easy-to-use and hard to break in order to make commercialization of this specimen holder possible.  
  
*Additional Comments to Authors:*  
*Additional references suggested:  
The prototype of the specimen holder was described in:   
Bastian, B.L. and Fain, G.L. (1979). Light adaptation in toad rods: requirement for an internal messenger which is not calcium. Journal of Physiology 297: 493-520.  
  
The double-flash technique for isolating cone ERG responses (now attributed to Heikkinen et al., 2008) was developed in:   
Koskelainen, A., Hemilä, S. & Donner, K. (1994). Spectral sensitivities of short- and long-wavelength sensitive cone mechanisms in the frog retina. Acta Physiologica Scandinavica 152, 115-124.*

Thank you for reminding us of these important references, they have been now added to the manuscript.